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(54) Title: METHOD FOR INTRODUCTION OF MOLECULES INTO CELLS

(57) Abstract: The invention provides a method of eliciting an immune system response in a mammal, which method comprises: injecting a first antigen into a first injection site in skeletal muscle in said mammal; optionally injecting a polynucleotide functionally encoding a second antigen into a second injection site in skeletal muscle in said mammal; positioning electrodes in said skeletal muscle such that current travelling between said electrodes passes through said first and/or second injection site; and electrically stimulating said skeletal muscle with an electrical current between said electrodes having a field strength in said skeletal muscle of from 10 to 300 V/cm whereby to assist in cellular uptake of said first antigen and/or said polynucleotide.

METHOD FOR INTRODUCTION OF MOLECULES INTO CELLS

The present invention relates to a method for eliciting an immune response in an animal by injection of an antigen (e.g. a protein, peptide, nucleic acid or other molecule) into skeletal muscle, as well as to the use of an antigen for the preparation of an immunization composition for use in such a method.

Scientists are continually discovering antigens (e.g. proteins, polypeptides, protein-epitope conjugates, and other molecules) capable of generating desirable immune system responses. Despite these new discoveries, a major obstacle facing the medical profession is how to safely deliver effective quantities of these antigens to patients to create the desired immune system response, e.g. for immunization or antibody production.

Currently, most antigens are delivered orally or intravenously. Oral and intravenous delivery methods, however, have several shortcomings. First, a large percent of orally or intravenously delivered agents are degraded by the body before arriving at the target organ or cells. Acids and enzymes in the stomach and intestine, for example, can break down many antigens, particularly proteins and oligo and polypeptides. Additionally, intravenously delivered antigens are often sequestered by the liver or kidney before arriving at the desired cells. Second, oral and intravenous delivery is non-specific. That is, the antigen is delivered to both target and non-target cells.

Skeletal muscle is a promising candidate for antigen delivery. First, skeletal muscle constitutes over 50% of a human's body mass, most of which is easily accessible compared to other tissues and organs of the body. Secondly, muscle is an ideal site for genetic

immunization because it is easily accessible and proteins made in the muscle are secreted, thus eliciting an immune response. Finally, skeletal muscle cells are non-dividing. Therefore, skeletal muscle cells are capable of expressing a protein coded by a gene for a longer time period than would be expected of other cell types that are continually dividing. Because the protein is expressed for a longer time, fewer treatments would be necessary.

There are several methods known in the art for transferring bioactive agents into skeletal muscle, such as intramuscular injection. The clinical applicability of direct muscle injection, however, is limited mainly because of low transfection efficiency, typically less than 1% transfection efficiency. It has been demonstrated that the efficacy of transfection can be improved if injections are done in regenerating muscle. Regeneration is induced some days (e.g. three days) before injection with the drug Bivucain. While injection in regenerating muscles induced by Bivucain show higher efficiency, the method has limited applicability in humans because of the severe damage caused to the muscle.

Methods of cell transfection also suffer from the difficulty that they must often be carried out under general anaesthetic. This is because certain local anaesthetics, such as Marcain, cause muscle cell death and so reduce transfection efficiencies. The use of general anaesthetic causes undesirably increased risks in human patients and is more difficult and costly to carry out than local anaesthesia in large animals such as sheep, coats and cattle.

From the foregoing, it will be appreciated that there is a continuing need in the art for improved methods of delivering antigens.

We have now found that antigen delivery for immune response elicitation can particularly effectively be

achieved using electroporation of skeletal muscle.

Thus viewed from one aspect the invention provides a method of eliciting an immune system response in a mammal, e.g. a method of immunizing a mammal, which method comprises:

injecting a first antigen, e.g. in an immune response eliciting amount, into a first injection site in skeletal muscle in said mammal;

optionally injecting a polynucleotide functionally encoding a second antigen into a second injection site in skeletal muscle in said mammal;

positioning electrodes in said skeletal muscle such that current travelling between said electrodes passes through said first and/or second injection site; and

electrically stimulating said skeletal muscle with an electrical current between said electrodes having a field strength in said skeletal muscle of from 10 to 300 V/cm, i.e. such that the mean potential gradient between the electrodes in the muscle is 10 to 300 V per cm, whereby to assist in cellular uptake of said first antigen and/or said polynucleotide.

In the method of the invention, the first and second injection sites may be the same or different, the first and second antigens may be the same or different, and injection of the first antigen and the polynucleotide may be simultaneous or separate (locationally and/or temporally). It is preferred however that the first and second injection sites are within the same muscle, it is also preferred that the first and second antigens share a common antigenic epitope, and it is further preferred that the first antigen is injected at least 24 hours prior to the polynucleotide.

By functionally encoding it is meant that the polynucleotide, optionally together with a further administered polynucleotide, should enable a cell transfected therewith to express the second antigen. To

this end, the polynucleotide should possess (e.g. in the known manner) appropriate start and stop sequences and that it or a further polynucleotide should functionally encode any enzymes required for expression of the second antigen that are not normally expressed by the mammalian cells. By polynucleotide is meant RNA or, more preferably, DNA, especially cDNA, optionally in the form of a plasmid. It will be appreciated that DNA encoding an antigen does so indirectly, i.e. via the corresponding RNA sequence.

in a further aspect the invention provides a method of enhancing an immune response of a mammal comprising:

injecting an antigen into a first injection site in the skeletal muscle of the mammal;

positioning electrodes near the first injection site such that current travelling through the electrodes passes through the first injection site;

electrically stimulating the muscle with a first electrical current having a field strength of from about 10 V/cm to about 233 V/cm;

injecting a DNA molecule coding for the antigen into a second injection site in the skeletal muscle of the mammal;

positioning electrodes near the second injection site such that current travelling through the electrodes passes through the second injection site; and

electrically stimulating the muscle with a second electrical current having a field strength of from about 10 V/cm to about 233 V/cm; wherein the DNA molecule is injected at a time of from about one week to about several years after the injection of the antigen.

Viewed from a further aspect the invention provides the use of an antigen and/or a polynucleotide functionally encoding an antigen for the manufacture of pharmaceutical compositions for use in a method of eliciting an immune response according to the invention.

Viewed from a still further aspect the invention

provides a pharmaceutical composition for use in a method of eliciting an immune response according to the invention, said composition comprising an antigen and/or a polynucleotide functionally encoding an antigen, together with a physiologically tolerable carrier or excipient.

Viewed from a yet still further aspect the invention also provides an antigen and/or a polynucleotide functionally encoding an antigen for use in a method of eliciting an immune response according to the invention.

In one embodiment, the method of the invention comprises a method of immunizing a mammal with an antigen comprising:

injecting the antigen into an injection site in the skeletal muscle of the mammal;

positioning electrodes near the injection site such that current travelling through the electrodes passes through the injection site; and

electrically stimulating the muscle with an electrical current having a field strength of from 10 V/cm to 300 V/cm. In a further embodiment, the method comprises a method of inducing a cellular immune response in a mammal comprising:

injecting DNA encoding an antigen into an injection site in the skeletal muscle of the mammal;

positioning electrodes near the injection site such that current travelling through the electrodes passes through the injection site; and

electrically stimulating the muscle with an electrical current having a field strength of 10 V/cm to 233 V/cm.

We have surprisingly found that the method of the invention may be used to transfect not just skeletal muscle cells but a significant number of other cell types and hence that it is especially, and surprisingly, applicable for eliciting the T-cell and B-cell immune

responses or for antigen delivery rather than simply for delivery of DNA. In particular we have found that the immune response is potentiated by using a proteinaceous antigen and using a polynucleotide functionally encoding an antigen possessing a common antigenic epitope.

Viewed from a further aspect the invention thus provides the use of a polynucleotide functionally encoding an antigen for the manufacture of a pharmaceutical composition for use in a method of treatment of a mammal to induce a cellular immune response, e.g. an immune response, in the lymphatic system, which method comprises injecting said composition into an injection site in skeletal muscle of said mammal and electroporating cells around said injection site, e.g. using the techniques described herein.

Viewed from a further aspect the invention thus provides the use of an antigen for the manufacture of a pharmaceutical composition for use in a method of treatment of a mammal to induce a humoral immune response, e.g. an immune response in the lymphatic system, which method comprises injecting said composition into an injection site in skeletal muscle of said mammal and electroporating cells around said injection site, e.g. using the techniques described herein.

Viewed from a further aspect the invention also provides a method of inducing a humoral immune response in a mammal comprising:

injecting an antigen into an injection site in the skeletal muscle of the mammal;

positioning electrodes near the injection site such that current travelling through the electrodes passes through the injection site; and

electrically stimulating the muscle with an electrical current having a field strength of about 10 V/cm to about 233 V/cm.

Viewed from a further aspect the invention provides a pharmaceutical composition comprising a proteinaceous antigen and cell-free polynucleotide (e.g. a plasmid) functionally encoding a protein antigen, optionally and preferably together with a physiologically acceptable carrier or excipient, preferably wherein said proteinaceous and protein antigens include at least one antigenically equivalent epitope.

Viewed from a yet still further aspect the invention provides a kit comprising: a first pharmaceutical composition comprising a proteinaceous antigen optionally and preferably together with a physiologically acceptable carrier or excipient; and a second pharmaceutical composition comprising cell-free polynucleotide (e.g. a plasmid) functionally encoding a protein antigen, optionally and preferably together with a physiologically acceptable carrier or excipient, preferably wherein said proteinaceous and protein antigens have at least one antigenically equivalent epitope.

Viewed from a yet still further aspect the invention provides the use of an antigenic substance and/or a polynucleotide functionally encoding an antigen for the manufacture of a pharmaceutical composition for use in a method of introducing said antigenic substance and/or said polynucleotide into non-muscle cells which comprises injecting said composition into an injection site in skeletal muscle of a mammal and electroporating cells around said injection site, e.g. using a technique as described herein.

The method of the invention is thought to be similar to conventional electroporation in its transfection effect. Electroporation works on the principle that a cell acts as an electrical capacitor and is generally unable to pass current. Subjecting cells to a high-voltage electric field, therefore, creates transient permeable structures or micropores in

the cell membrane. These pores are large enough to allow antigens, polyaminoacids, pharmaceutical drugs, polynucleotides, and other polar compounds to gain access to the interior of the cell. With time, the pores in the cell membrane close and the cell once again becomes impermeable.

Conventional electroporation, however, employs high field strengths from 400 V/cm to several kV/cm. In contrast to conventional electroporation, the field strength used in the present invention ranges from 10 V/cm to 300 V/cm. These lower field strengths are thought to cause less muscle damage without sacrificing, and indeed increasing, transfection efficiencies. Furthermore, using the method of the present invention, transfection efficiencies can be tightly regulated by altering such parameters as frequency, pulse duration and pulse number.

Transfection by the method of the present invention also allows effective immunisation using antigens, or the combination of antigens and polynucleotides encoding antigens (either simultaneously, or sequentially in any order).

The increase in transfection efficiency is observed if the muscle is electrically stimulated immediately, or shortly after the injection. Thus, the semipermeable quality of the tissue induced by the stimulation is reversible. Moreover, it is dependent on current through the muscle; activity induced through the nerve does not affect transfection efficiency. Similarly, the enhanced immune response provided by the current method occurs when the muscle is electrically stimulated immediately, or shortly after injection.

Once transfected with the polynucleotide, the cells in the injection site are able to express the proteins coded by the nucleic acid. Therefore, the transfection method of the present invention can be used, for example, to transfect expression vectors for genetic

immunization (e.g. DNA vaccines).

In laboratory experiments involving DNA transfection into small mammals, it is usual to administer a general anaesthetic so as to avoid any unnecessary suffering. In human patients and in large animals such as cattle, however, general anaesthesia is not preferred. In order to reduce pain that may be associated with the method of the present invention, without the need for general anaesthesia, a local anaesthetic can be injected at the site of treatment prior to or in conjunction with the injection of the antigen or DNA. For example, in one embodiment of the current invention, the antigen or DNA may be mixed with Marcain, a local anaesthetic, followed by electroporation. This is unexpected because Marcain is known to cause muscle cell death and so would be expected to greatly reduce transfection efficiency.

This injection of a local anaesthetic is surprisingly effective in methods involving electroporation to promote cellular uptake of injected substances and forms a further aspect of the invention. Viewed from this aspect the invention provides a method of delivering a molecule to the skeletal muscle of a mammal *in vivo* comprising:

injecting a local anaesthetic into the skeletal muscle of the mammal;

injecting a molecule into an injection site in the skeletal muscle;

positioning electrodes near the injection site such that current travelling through the electrodes passes through the injection site; and

electrically stimulating the muscle with an electrical current having a field strength of from 10 V/cm to 300 V/cm. In a particularly preferred embodiment this method comprises a method of delivering DNA molecule to the immune system of a mammal *in vivo* comprising:

mixing a first solution comprising the DNA molecule with a second solution comprising a local anaesthetic to produce a DNA-anaesthetic mixture;

injecting the DNA-anaesthetic mixture into an injection site in a skeletal muscle of the mammal;

positioning electrodes near the injection site such that current travelling through the electrodes passes through the injection site; and

electrically stimulating the muscle with an electrical current having a field strength of from 10 V/cm to 300 V/cm.

Embodiments of the invention will now be described further with reference to the following non-limiting Examples and the accompanying drawings, in which:

Figure 1 - graphically illustrates a method of delivering antigens and DNA into skeletal muscle according to the present invention.

Figure 2 - is a graphical illustration of an electrical stimulation delivered according to the method of the present invention;

Figure 3 - is an image of cells stained with anti-agrin polyclonal antibodies derived from a rabbit genetically immunized with an expression vector coding for rat agrin using the stimulation technique of the present invention;

Figure 4 - are graphs illustrating improved genetic immunization of mice and rats using the stimulation technique of the present invention versus naked DNA injection;

Figure 5 - are graphs illustrating relative amounts of specific subtypes of antibody reactive with given antigens at four and eight weeks after immunization;

Figure 6 - is a bar graph illustration of mean luciferase activity in lymph nodes of mice after transfection of muscle with Luc cDNA;

Figure 7 - is a bar graph illustration of mean luciferase activity in lymph nodes of rats after

transfection of muscle with Luc cDNA;

Figure 8 -are graphs illustrating IgG1 antibody levels in mice at 4, 8, and 9 weeks after genetic immunization;

Figure 9 - are graphs illustrating IgG2a antibody levels in mice at 4, 8, and 9 weeks after genetic immunization;

Figure 10 -are graphs illustrating IgG2b antibody levels in mice at 4, 8, and 9 weeks after genetic immunization;

Figure 11 - is a bar graph illustrating luciferase activity in muscle cells 5 days after transfection with Luc plasmid DNA;

Figure 12 - is a bar graph illustrating mean luciferase activity in mouse muscles after a second immunization with 85B and luciferase cDNA (a low value indicates a strong cellular immune response and efficient killing of transfected cells);

Figure 13 - are graphs illustrating antibody levels after protein immunization (a high level of IgG1 indicates a humoral immune response);

Figure 14 - are graphs illustrating antibody levels in mice eight weeks after genetic immunization with low electrical field strength;

Figure 15 - are graphs illustrating the responses of T-cells from immunized animals to the indicated peptides;

Figure 16 - are fluorescence micrographs showing (a) cells expressing green fluorescent protein after transfection by the method of the invention and (b) muscle-resident Adipocytes expressing the S-100 protein;

Figure 17 - are fluorescence micrographs showing (a) cells expressing green fluorescent protein after transfection by the method of the invention and (b) muscle-resident connective tissue cells expressing the IgG protein thy-1; and

Figure 18 - are fluorescence micrographs showing

(a) cells expressing green fluorescent protein after transfection by the method of the invention and (b) muscle-resident connective tissue cells expressing the intracellular protein vimentin.

Figure 19 - are graphs indicating the response of goats to transfection with DNA under sedation and local anesthetic, with and without electroporation (EP).

The present invention is directed to a novel method for administering an antigen, and optionally also a polynucleotide encoding for an antigen. The method of the present invention involves passing an electrical current through the skeletal muscle tissue. Unlike previously described electroporation methods, however, the method of the present invention can be effected with a low field strength and thus causes much limited tissue damage. Other parameters such as the number of pulse trains, frequency, pulse number and pulse duration can be varied in order to regulate the amount of antigen or polynucleotide delivered.

While the antigen administered in the method of the invention may be any antigenic molecule, it is preferably a polar species and more preferably it is peptidic, e.g. a synthetic oligo- or polypeptide, a protein-epitope conjugate, a protein or an antigenic protein fragment, for example an aglycosylic oligopeptide corresponding to an antigenic fragment of a protein of interest. The antigen may be administered in order to stimulate an immune response so as to confer immunity to the antigen to the mammal or alternatively to provoke antibody production, e.g. for commercial use. Especially preferably, the antigen is a protein or protein fragment, optionally an aglycosylic fragment. Where an antigen "A" and a polynucleotide encoding an antigen "B" are both administered, it is preferred that antigen "A" is peptidic and that antigen "B" should comprise a peptidic sequence having a high degree of homology with at least one epitope of antigen "A", e.g.

at least 80% homology, more preferably at least 90% homology (e.g. over a sequence of at least 20 amino acids, discounting deletions). More especially antigens "A" and "B" are identical, or antigen "A" should comprise the entire sequence (or at least 90% of the entire sequence) of antigen "B" or antigen "B" should comprise the entire sequence (or at least 90% of the entire sequence) of antigen "A".

Alternatively, the first antigen and the second antigen may conveniently be different surface proteins (or fragments thereof) of the same viral, bacterial or fungal species.

As illustrated in Figure 1 of the accompanying drawings, in the method of the invention, generally, skeletal muscle is exposed and a predetermined amount of a molecule is injected into the muscle. In one embodiment the antigen or polynucleotide is dissolved in 0.9% sodium chloride (NaCl) or another physiologically tolerable aqueous vehicle. The exact solvent, however, is not critical to the invention. For example, it is well known in the art that other materials such as sucrose are capable of increasing molecular uptake in skeletal muscle. Other substances may also be co-transfected with the molecule of interest for a variety of beneficial reasons. For example, P188 (Lee, et al. PNAS., *4524-8*, 10, 89 (1992)), which is known to seal electropermeabilized membranes, may beneficially affect transfection efficiencies by increasing the survival rate of transfected cells.

With continued reference to Figure 1, electrodes are placed on the muscle, about 1-4 mm apart, near the area where the antigen or polynucleotide molecule was injected. The exact position or design of the electrodes is not critical, however it is preferred that current pass through the muscle fibers perpendicular to their direction of orientation in the area of the injected molecule.

With the electrodes in position, the muscle is electroporated or stimulated. As illustrated in Figure 2, the stimulation is preferably delivered as a square bipolar pulse having a predetermined amplitude and duration. In order to optimize the transfection efficiencies, these parameters have been widely varied and transfection efficiencies compared. For example, the voltages have ranged from approximately 0 to 50 volts; the pulse durations have ranged from 5 μs to 5 ms; the number of pulses have ranged from a single pulse to 30,000 pulses; and the pulse frequency within trains have ranged from 0.5 Hz to 1000 Hz.

The conclusion from these results is that so long as the field strength is above 10 V/cm, preferably above 50 V/cm the other parameters may be varied depending on the experimental conditions desired. While no upper limit was detected, effective transfection efficiencies were observed with much higher field strengths. The field strength of the stimulation can be calculated using the formula:

$$E=V/(2r\ \ln(D/r))$$

which gives the electric field between wires if D »r. Typically, in the formula, V= voltage = 10 V, D = distance between wire centers = 0.1-0.4 cm, r diameter of electrode = 0.06 cm. (See Hofhiann, G. A. "Cells in electric fields", in E. Neumann, A. E. Sowers, & C. A. Jordan (Eds.), "Electroporation and electrofusion in cell biology" (pages 389-407). Plenum Publishing Corporation (1989)). At 10 volts, the field strength is between 163 V/cm - 43 V/cm (from 0.1 to 0.4 cm between electrodes, respectively).

Because D is not much greater than r, it may be more appropriate to use the formula for electric fields between large parallel plates:

$E=V/D$

This gives a similar field strength of between 100 V/cm - 25 V/cm (from 0.1-0.4 cm between electrodes, respectively). It will be appreciated that the field strength, as well as other parameters, are affected by the tissue being transfected, and thus optimal conditions may vary. Using the parameters given in the present invention, however, optimal parameters can be easily obtained by one skilled in the art.

The electrical stimulator used for the experiments was reported below manufactured by FHC (Brunswick, ME 04011). Both Pulsar 6bp and the Pulsar 6bp-a/s stimulators have been used. The Pulsar 6bp-a/s delivers a maximal voltage of 150 V and a maximal current of 50 mA. The maximal voltage that can be delivered requires a resistance between the electrodes of greater than 3000 Ohms. The stimulators have been operated at constant voltage mode. Because of the low resistance in the muscle, the voltages have been lower in the Examples below. In all experiments the current has been maintained at 50mA.

It will be appreciated by one skilled in the art that numerous electrode configurations can be employed.

It will be appreciated that any transcribable or translatable polynucleotide can be used with the method of the present invention, for example, plasmid DNA, linear DNA, antisense DNA and RNA. In one embodiment, the nucleic acid is a DNA expression vector of the type well known in the art. Generally, an expression vector contains a promoter operably linked to a DNA molecule that codes for the protein of interest followed by a termination signal such as a polyadenylation signal. Other elements required for bacterial growth and proper mammalian processing may be included, such as the β-lactamase coding region, an ft origin and ColE 1-derived plasmid replication origin. Similar constructs

containing a DNA coding region of interest can be constructed by one skilled in the art.

Various protein and polyaminoacid antigens may effectively be employed in the present method. In one preferred embodiment an immune response is generated by administration of a protein antigen and the corresponding cDNA, both by the method of the invention, either simultaneously or sequentially in either order. When administered sequentially, the period between administrations is typically 1 day-20 weeks (e.g. 2-16 weeks), particularly 4-8 weeks. The combination of DNA and antigen may also be used by administration of DNA by the method of the invention, either followed or preceded by injection of an antigen without electrical stimulation. Such an antigen may be, for example, a protein or a line or inactivated virus.

As illustrated in the examples below, molecules other than antigens and antigen-coding nucleic acids can be delivered to the muscle using the technique of the present invention. Thus for example nucleic acid and non-antigenic proteins can be simultaneously introduced. In one embodiment, the large T-antigen nuclear localization signal (a protein) is mixed with a plasmid containing the DNA coding region for an antigenic protein. The large T-antigen nuclear localization signal is a protein that binds DNA and facilitates its transport into the nucleus of a cell. In other systems, large T-antigen nuclear localization signal has been shown to increase transfection efficiency. Using the method of the present invention, large T-antigen nuclear localization signal also increases the transfection efficiency of antigen-encoding DNA.

The method of the present invention can particularly advantageously be used to drive the immune response of an animal in a specific direction. For example, DNA encoding for an antigen was administered to a group of mice according to the method of the

invention. After four and eight weeks, serum was collected from the mice and antibodies analyzed by ELISA. The mice had a high level of IgG2a antibodies that reacted with the antigen, indicating that a strong cellular immune response was induced. When animals were immunized according to the method, increased numbers of CD8+ and CD4+ T-cells that secreted interferon gamma were measured with ELISPOT after stimulation with peptides specific for MIHC I and II binding. This demonstrates a strong cellular immune response as well as induction of Th-1 cells. When animals were given a boost injection in combination with a reporter gene a reduced expression of reporter gene (indicating a stronger cellular immune response) was observed in animals that had been immunized according to the present invention.

Another example is to drive the immune response in the other direction with preferential stimulation of the humoral branch of the immune system. When protein antigens were administered in accordance with the present invention, higher IgG1 and IgG2b antibody titers could be detected. When these protein-treated animals were given a boost injection of antigenic protein in combination with a reporter gene, increased expression of the reporter gene was observed. These results indicate that immunization with antigenic protein in combination with electrical stimulation had altered the immune response in such a way as to make the animals tolerant to the antigen. Alternatively, such immunization may stimulate an immune reaction that is not efficient in killing muscle cells. This method may be useful for the treatment of various autoimmune diseases in which a strong cellular immune response causes or contributes to the disease.

In a preferred embodiment, the present invention thus provides a method of controlled stimulation (and/or modulation) of the cellular and/or humoral branch of the

mammalian immune system by administration of one or more doses of antigen and/or the corresponding DNA (especially cDNA) to muscles by injection and electrical stimulation, as described above. Where more than one administration is made, this may be of antigen and/or DNA, simultaneously or sequentially in order to provide the desired combination of cellular and/or humoral response, as illustrated below.

One mechanism which explains these results is that other cells in addition to skeletal muscle cells are transfected by the method of the present invention. Such cells may include lymphocytes, macrophages, dendritic cells, etc. For example, animals were intramuscularly injected with Luc cDNA. The muscles were electrically stimulated shortly after the injection. Animals were sacrificed at two and seven days, and their spleens and lymph nodes removed and analyzed for luciferase activity. As shown in Table 1 below and Figures 6 and 7, a large increase in luciferase activity in the lymph nodes and spleen was found. These findings indicate that immune cells residing within the muscle are transfected by the method of the present invention.

In a further preferred embodiment, the invention therefore provides a method of transfecting non-muscle cells residing within skeletal muscles with nucleic acids (especially DNA and preferably cDNA), antigens (particularly protein and other polyaminoacid antigens) and other molecules by the injection and electrical stimulation method as described above. In particular, a method is provided for inducing an immune response in a mammal by transfection of immune cells (such as lymphocytes, macrophages and/or dendritic cells) resident in skeletal muscles by inducing the uptake into such cells of DNA (preferably cDNA), antigens (preferably protein antigens) and/or other molecules by infection and electrical stimulation as described above.

Preferably, transfection of muscle-resident non-muscle cells occurs to an extent at least as great and preferably greater than the extent of muscle cell transfection. This may be achieved, for example, by the use of low field strengths such as field strengths below 100 V/cm, particularly 5-100 V/cm and especially 5-25 V/cm.

Local anaesthetics are frequently used in medical procedures to reduce the pain and anxiety caused by the procedure and are greatly preferable to general anaesthetics for use in human patients and in larger animals, such as cattle, sheep and goats. In humans the use of general anaesthetic carries increased risks and for large animals the procedure can be difficult and time consuming. Unfortunately, some local anaesthetics are known to cause muscle cell death and so are typically not suitable for use with muscle transfection techniques. Marcain (2.5 mg/ml from Astra, *bupivacain hydroclorid)* is one such local anaesthetic. A structurally similar local anesthetic is lidocain and an alternative used in the examples below is ketaime. Marcain may be mixed with DNA to reduce the possible discomfort of muscle stimulation associated with electroporation. As seen in Figures 8 through 11, the administration of Marcain in combination with the methods of the current invention had no significant effect on either the resulting immune response or the transfection efficiency, despite its known tendency to induce muscle-cell death. For this procedure, a high concentration of Marcain was used without adverse effects on the efficiency of the method. However, it would be appreciated by those of skill in the art that other concentrations of Marcain and other anaesthetics can be used and a suitable concentration may be established by standard techniques.

One may transfect the cells residing in the skeletal muscle using low electrical field strength,

e.g. less than approximately 100 V/cm. In certain embodiments, cells within the muscle are transfected with a field strength of at least about 5 V/cm. In certain other embodiments, cells within the muscle are transfected with a field strength of at least about 10 V/cm. For example DNA encoding an antigen was administered according to the method of the invention and the muscle was stimulated with a field strength of about 10 V/cm to about 25 V/cm. As seen in Figure 14, a large increase in antibodies against the antigen were detected for low voltage stimulation as compared to injected naked DNA. One might also use much greater field strengths. For example, one could transfect cells within muscle using field strengths in the range of from approximately 10 V/cm to approximately 300 V/cm. In certain embodiments of the present invention, one could transfect cells within muscle using field strengths of from approximately 12 V/cm to approximately 175 V/cm; from approximately 125 V/cm to approximately 233 V/cm; or from approximately 10 V/cm to approximately 233 V/cm.

One skilled in the art would appreciate that a boost injection (immunization) given subsequent to the first immunization is likely to enhance the immune response further. This can be done with electroporation or with other immunization strategies and may comprise the injection of an antigen, of DNA or of both. For example, animals have been immunized with plasmid DNA and then later with a certain virus encoding the same antigen in order to obtain a further increase in the cellular immune response. See, e.g., Schneider et al., *Nature Medicine* 4:397 (1998). The boost injection may be given soon after the initial immunization (i.e., within a few days or a week). One skilled in the art would also appreciate that booster immunizations may be given many years after the first injection. In certain embodiments of the present invention, boost injections are given at a time from about two weeks to about four

months after the initial immunization. In certain preferred embodiments, boost injections are given at a time from about one month to about two months after the initial immunization.

Without being bound by theory we have several possibilities for why electroporation enhances the immune response; first electroporation enhances expression of the antigen; second it enhances expression in other (i.e. non-muscle) cells that are later found in lymph nodes or spleen; third it seems to enhance the muscle cells' ability to present antigen (MHC class I staining); fourth, electrical stimulation will cause muscle activity that is likely to increase the lymph flow from the muscles (e.g. low voltage stimulation). The mechanism is probably due to many of these factors in combination and the contribution of each one of them will depend on the type of immune response being induced. This may be controlled by use of an appropriate embodiment of the invention.

As mentioned above, it has surprisingly been found that electroporation of cells at the injection site of a molecule in skeletal muscle is effective even at surprisingly low field strengths of 5 to 25 V/cm, e.g. 5 to 24 V/cm, preferably at least 10 V/cm e.g. 10-20 V/cm. This forms a further aspect of the invention. Viewed from this aspect the invention provides a method of delivering a molecule to cells residing within the skeletal muscle of a mammal *in vivo* comprising:

injecting the molecule into an injection site in the skeletal muscle of the mammal;

positioning electrodes near the injection site, such that current travelling through the electrodes passes through the injection site; and

electrically stimulating the muscle with an electrical current having a field strength of at least 5 and less than 25 V/cm.

Thus is also provided in an further aspect of the

invention a method of genetically immunizing a mammal comprising:

injecting DNA encoding an antigen into an injection site in a skeletal muscle of the mammal;

positioning electrodes near the injection site such that current travelling through the electrodes passes through the injection site; and

electrically stimulating the muscle with an electrical current having a field strength of less than about 100 V/cm.

In a further aspect the invention also provides a method of delivering a molecule to cells residing within the skeletal muscle of a mammal *in vivo* comprising:

injecting the molecule into an injection site in the skeletal muscle of the mammal;

positioning electrodes near the injection site, such that current travelling through the electrodes passes through the injection site; and

electrically stimulating the muscle with an electrical current having a field strength of from about 10 V/cm to about 233 V/cm.

Example 1 - Large T Antigen Nuclear Localization Signal:

Wistar rat muscles were injected with DNA plasmid containing the f3-galactosidase gene containing a 100:1 molar excess of large T-antigen nuclear localization signal. This has been shown in other transfection studies to improve the transfection. *See,* Collas et al. *Transgenic Res.* 6:45 1-8 (1996). The muscle were stimulated with 10 trains of 100 pulses of 50 p.s duration. The muscles containing the large T-antigen nuclear localization signal had the highest number of transfected fibers. Specifically, the muscle co-transfected with large T-antigen nuclear localization signal had 100 and 38 transfected fibers versus 7.3 and 4.7 for the muscles transfected only with DNA, respectively. These data illustrate that transfection

efficiencies can be aided by mixing the DNA with non-nucleic acid molecules. In addition, this data illustrates that non-nucleic acid molecules can also be delivered to the muscle using the electroporation techniques of the present invention. No improvement was seen in cells that were not stimulated following injection.

Example 2 - Genetic Immunization of Rabbits:

A female rabbit (4.5 kg) was injected into the right femuralis rectus with 2 milliliters of 1 $\mu g/\mu l$ of DNA plasmid containing the rat neural agrin cDNA driven by the CMV promotor (Cohen et al. MCN, 9, 237-53 (1997)). The first milliliter was injected equally in ten places superficial in the muscle followed by 10 trains of 1000 pulses delivered at a frequency of 1000 Hz. The second milliliter was placed further down in the muscle. To test the rabbit serum, rat muscles and COS cells were transfected with the same construct. Muscles were taken out 5 days after transfection and the COS cells were stained 4 days after transfection.

Bleeds were collected at days 0, 19, 50, 81 and 106 and diluted 1:100 and 1:1000. After 19 days the bleed contained enough antibody in the serum to give a weak staining of transfected fibers when diluted 1:10. As a positive control the monoclonal antibody (mAb) AG-86 was used. *See* Hoch et al. *EMBOJ*, 12 (13): 2814-21(1994). Preimmune serum did not show any staining of transfected fibers. Later bleeds all had agrin antibodies in the serum. Bleed collected at day 50 or later contained sufficient antibodies to stain sections at a dilution of 1:1000.

Figure 3a illustrates the agrin transfected COS cells stained with antiserum from immunized rabbit (diluted 1:100) and fluorescein conjugated secondary antibody. COS cells were stained by first fixing the cells in 1.5% paraformaldehyde for 10 minutes, followed

by a 30 minute wash with phosphate buffered saline (PBS). The cells were then blocked with 0.2% bovine serum albumin, triton X-100, 0.1% in PBS 0.1M, for 4 minutes. Serum diluted in the same solution was added to the cells and allowed to incubate for 20 minutes. Cells were washed for 4 minutes in PBS and incubated with the secondary antibody (Cappel, 55646) for 10 minutes followed by a PBS wash. Mouse primary mAb Agr-86 was included in the same antibody mixture and rhodamin conjugated secondary antibody (Sigma T-5393, St. Louis. MO) was used at a dilution of 1:100. Figure 3b illustrates the same cells stained with mAb Ag-86/rhodamin conjugate. These data illustrate the potential of the technique of the present invention for genetic immunization or for DNA vaccine technology.

Example 3 - Genetic Immunization of Mice:

Groups of two-month old male Sprague Dawley rats were inoculated bilaterally in the EDL and soleus muscles with a total of 200 micrograms (4 x 50 microliters of a 1 mg/ml solution of DNA in saline) of three different eukaryotic expression vectors containing the cytomegalovirus immediate early promoter (CMV) and the coding sequences for the following proteins: DH-CNTF, an agonistic variant of human ciliary neurotrophic factor (Saggio et al. EMIBO J. 14, 3045-3054, 1995); AADH-CNTF, an antagonistic variant of human ciliary neurotrophic factor (Di Marco et al. Proc. Natl. Acad. Sci. USA 93, 9247-9252, 1996); sec-DHCNTF, a secreted form of DH-CNTF. The muscles were either not electrically stimulated or stimulated immediately after DNA injection using 30 trains of 100 or 1000 square bipolar pulses (duration 200 microseconds; amplitude setting 150 V, effective voltage ~25 V) each, delivered at a frequency of 1000 Hz with a two second interval between successive trains.

Groups of two-month old male CD1 mice were

inoculated bilaterally in the quadriceps muscles with 100 micrograms (2 x 50 microliters of a 1 mg/ml solution of DNA in saline) of sec-DHCNTF plasmid, with or without electrical stimulation of the muscle immediately after DNA injection. Stimulation conditions were 10 trains of 1000 square bipolar pulses (amplitude setting 150 V) delivered at a frequency of 1000 Hz with a two second interval between successive trains.

Blood was collected from the retroorbital sinus at selected time points and serum was prepared and stored at -20°C. The presence of anti-CNTF antibodies in rat and mouse sera was determined by ELISA. Microtiter plates coated with recombinant human CNTF were incubated with serial dilutions of sera, followed by alkaline phosphatase-conjugated antibody against rat or mouse IgG (Pierce). The plates were then incubated in the presence of p-nitrophenyl-phosphate and the absorbance at 405 nm was determined using a microplate reader. Antibody titers were defined as the dilution of serum producing an absorbance reading equal to 50% of that obtained with a saturating concentration of anti-CNTF antiserum.

The results are shown in Figure 4. Titers could not be averaged with precision, due to the fact that some animals did not develop detectable amounts of antibody. Data are therefore presented for individual animals, with a value of 1:100 representing a low or undetectable antibody titer (reciprocal titer 3/4 100). The results were similar for all plasmids used, as well as for rats and mice, as depicted in Figure 4. Similar results were also obtained in both rats and mice with another plasmid encoding an unrelated viral protein (data not shown). In both rats and mice, electrical stimulation immediately after DNA injection led to approximately 5 to 10-fold higher antibody titers than simple DNA injection. This was true for stimulation with both high and low numbers of pulses. These results

demonstrate that the electroporation method increases the efficiency of DNA-mediated immunization.

Example 4 - Materials and Methods for Genetic Immunization:

The materials and methods listed below were employed throughout Examples 5-14, except as otherwise indicated.

Protein Purification.

Both of the mycobacteria secreted proteins MPB70 and 85B were isolated and purified from culture fluid of *Mycobacterium bovis* BCG Tokyo and BCG Chopenhagen respectively, growing on Sauton media. PBS pH 7.4 was used to resuspend freeze-dried aliquots of the purified proteins to appropriate concentration for immunization or ELISA plate coating.

Plasmids.

The following antigen-expressing plasmids were used in these experiments: *CMV70* which is the M. *bovis* MPB70 protein encoding sequence inserted into *the pcDNA3*, a mammalian expression vector from Invitrogen (Carlsbad, California) with a CMV promoter (from Hewinson et al., Central Veterinary Laboratory, Surry, UK).

85b, which contains the mycobacterium tuberculosis gene encoding 85B without the mycobacterial signal sequence inserted into the *v1Jns-tPA* vector from Merck. In this plasmid, the bacterial gene is preceded by the promoter intron A of the first immediate early antigen *IE1* of CMV *(85b* from K. Huygen, Pasteur Institute of Brussels, Belgum).

Likewise, *85a* expresses the mycobacterium tuberculosis gene encoding the 85A protein, and a different form of 85b that it is inserted into the *VR1020* vector from VICAL (from K. Huygen, Pasteur Institute of Brussels, Belgum).

Plasmids from transfected *E. Coli* cultures were amplified and purified by using Genomed Jetstar purification kit, and by Aldevron DNA Purification Service to GMP standards. The purity of our DNA constructs were confirmed by enzyme digestion and agarose-EtBr electrophoresis. The concentration was measured by the 280/260 nm absorption ratio. The stock solution was stored at -20°C until needed.

The luciferase reporter gene used in these experiments contained a CMV promoter (VR-1255 from VICAL).

Luciferase assay.

The assay was performed using the kit developed by Promega, with the organs in question homogenized and added to the assay buffer and purified by centrifugation. Activity was with a TD-20/20, Luminometer from Turner Designs (Sunnyvale, CA, USA).

Animal experiments.

Six- to eight-week-old female B6D2 mice or BalbC mice were anesthetized and used in accordance with Norwegian rules for animal experiments.

Protein immunization.

Mice were immunized subcutaneously with 100 µl equal amount of proteins (MPB70 or 85B) at a concentration of 1 mg/ml in PBS sonicated with Incomplete Freunds Adjuvant (Behringwerke AG, Marburg, Germany).

BCG vaccination/immunization.

Mycobacterium bovis BCG (Moreau) was harvested from cultures grown in Satoun medium and washed twice with PBS buffer. The spun-down bacteria were homogenized carefully with PBS to an approximate concentration of 200 mg/ml. 100 µl of this suspension was injected

subcutaneously into the mice.

DNA injections and immunization with electrical stimulation (EP).

Intramuscular injections were given with a 28-gauge insulin needle to deliver 0.5 - 50 μg of plasmid DNA in 50 μ of physiological saline to quadriceps in mice bilaterally (final concentration of DNA was 0.01, 0.1, or 1 μg/μl, for a total of 1, 10 or 100 μg DNA per mouse). Following the DNA injection, electrodes were placed on the skin to deliver an electric field at the site of DNA delivery. The electroporation was given as 8 trains of 1000 pulses delivered at a frequency of 1000 Hz. Each pulse lasted for 200 μs positive and 200 μs negative for a total pulse duration of total 400 μs. The electrical field strength varied with the change in resistance in the tissue of each animal, but the field strength was in the range of approximately 25-35 V over approximately 2.5 - 3 mm, or from about 83 V/cm to about 140 V/cm. Each train was delivered at two second intervals, with each train lasting one second.

Serum sampling.

Venous blood was taken from the mice after four and eight weeks. Samples were left over night at 4 °C spun down and stored aliquoted at -20°C.

ELISA.

ELISA were performed in Costar high-bind microtiter plates coated with native protein (85B or MPB70) 100 μl per well, 5 μg/μl in PBS with sodium azide (stable for months). Plates were stored at least over night at 4°C before use. Before use and between every step, the plates were washed 3 times with PBS + 0.1 % Tween 20. All incubations were performed at 37°C for one hour, except the last developing step, which was performed at room temperature for 10 minutes. The assay steps were

as follows:

First, blocking was with PBS (without azide) containing 0.5% BSA, followed by application of serum samples diluted 27 or 64 times in PBS dilution buffer (0.2 % BSA and 0.2 % Tween 20. Biotinylated subtype-specific antibodies (anti-mouse IgG1 (clone A85-1), anti- mouse IgG2a (R19-15), both Lou Rat IgG1, and IgG2b (R12-3) rat IgG2a) (all three from Pharmingen) were added in a concentration of 0.5 μg/ml diluted in PBS dilution buffer. Streptavidin-HRP from Amersham diluted 1:1000 in PBS dilution buffer was then added. The amount of subtype-specific antibodies in serum were measured by OD at 405 nm after adding ABTS substrate in 0.1 M acetate buffer pH 4.0 with 3 % H_2O_2.

Normalization of OD values in subtype ELISA.

A positive control standard included in each ELISA microtiterplate was set to 1.0 (divided by itself). All other values obtained were divided by the positive control value, to be able to compare OD values from different microtiter plates within the same and different experiments.

Example 5 - Genetic Immunization with DNA Encoding Mycobacterial Antigens:

B6D2 mice were selected for the experiment and divided into three groups. One group received DNA plasmid and electrical stimulation (EP). The second group received only DNA. The third group consisted of control animals, which received only saline and electrical stimulation.

Each of the groups that received DNA were divided into subgroups according of the dose and type of DNA injected. The total DNA dose used in the mice was either 100, 10 or 1.0 μg in 100 μl saline (50 μl in each muscle). In Figure 5, the symbols refer to different doses of DNA, with each symbol representing the mean

titer from a group of mice (5-7 animals). Large symbols represent antibody titer from animals receiving 100 μg DNA; medium-size symbols, 10 μg DNA; and small symbols, 1 μg DNA. Circles represents EP-treated animals and diamonds no EP. Filled squares are from animals immunized with protein in IFA and plain lines (no symbols) are from animals immunized with BCG bacila. Serum samples were tested by an ELISA assay designed for subtyping of antigen (MPB70 or 85B) specific immunoglobulins. The average antibody titer is shown for each group of animals as a function of optical density at 405 nm with serial dilution of serum samples collected at 4 and 8 weeks. The overall pattern is that EP-treated animals react with a significantly higher titer of immunoglobulins to the antigen encoded by the injected plasmid for the three subclasses of immunoglobulin tested.

In mouse, a Th1 cellular immune response is indicated by elevated serum concentration of antigen-specific immunoglobulins of subclass 2a. A humoral Th2 immune response is characterized by increasing antigen-specific IgG1 and IgG2b antibodies in serum. Serum samples from DNA-EP immunized mice contained elevated levels of IgG1, IgG2a and IgG2b (Figure 5). This indicates that the animals react with both humoral and cellular immune responses. Compared with mice that have been immunized with *M bovis* BCG or protein-WA, our mice seem to have lower titers of IgG1 and IgG2b, but at the same or higher level with regard to IgG2a. These data indicate a strong Th1-associated cellular immune response when the animals are injected with DNA and EP-treated.

The *M tuberculosis-specific* secreted/membrane protein MPB70 tends to elicit a weaker immune response than the widely cross-reacting common mycobacterial antigen 85B. For *85b* DNA, all three doses of plasmid with EP give a high immunoglobulin response for the

three antibody subclasses tested. However, for *mpb 70* DNA, only the two highest doses of plasmid injected give a high immunoglobulin response. When the animals were injected with 1 μg of mpb70 DNA, we detected no immune response. Animals that receive more than 1 μg *85b* have almost the same antibody titer as an animal that were given 100 μg. For *mpb 70*, the response required a greater dose of DNA.

These results show that the dose of DNA injected into the animals can be reduced at least 100-fold and still give the same or higher immunoglobulin response against the antigen encoded. Without EP, none of the *mpb70* animals react against the antigen, but for *85b*, the two highest doses of DNA give an immune response, although it is significantly lower than the response in EP-treated animals. This might be because 85B is a common antigen, and the mice might have previously been exposed to it.

We have also studied the gene transfection efficiency by staining for the encoded antigen in frozen cross sections of the quadriceps muscle five days after plasmid DNA injection. By counting positive fibers in a defined area of the muscle section from EP-treated and non-treated animals, we found a nearly hundred-fold increase in antigen expression after EP (data not shown).

Muscle cells normally do not express either MHC class I or II at a detectable level. We have stained for both MHC I and II (mouse anti MHC class I from Pharmingen clone 34-2-12S, mouse anti MHC class II clone 25-29-17, both were directly conjugated with FITC) in frozen sections from the DNA-injected animals. With regard to MHC class I, we find that neither muscle cells nor other cells in the area express MHC I after injection of saline followed by EP. With injection only of plasmid (no EP), the cells in muscle fasciae start to express MHC I at a detectable level. When the animals

are injected with plasmid DNA followed by EP treatment, we see enhanced expression of MHC I in both the fasciae and on muscle fibers in the area where there are plasmid-transfected and gene-expressing fibers. Characteristic staining was found, with positive MHC I circular staining in the periphery of transfected fibers and their neighboring fibers, which is seen only in the plasmid transfected area.

MHC class II was detected in the fasciae and in-between muscle fibers only after plasmid injection followed by EP. HAS-staining shows that mononucleated cells are recruited to the area after DNA injection and EP treatment. The area in which we find MHC class II positive cells seems to be co-localized to that in which we find mononucleated cells after HAS staining. Without being bound by any particular theory, this co-localization may result from a combination of two factors. First, EP may cause local damage to the muscle. Second, the expression of a foreign antigen encoded by the injected plasmid functions as a strong signal for recruitment of immune cells.

Example 6 - Transfection of Immune Cells Residing in Skeletal Muscle of Rats:

Three rats were intramuscularly injected in the soleus muscle, surgically exposed, with 25 μg Luc cDNA dissolved in 50 μl of 150 mM sodium phosphate buffer pH 7.2. Following the injection, electrodes were inserted into the muscle near the site of the injection in two of the rats, and electroporation given. The third rat received no electrical stimulation.

After two days, the spleens of the three rats were removed and analyzed for luciferase activity. As shown in Table 1, the luciferase activity in the spleens of the rats that received electrical stimulation was more than ten times greater than the activity in the spleen of a rat that did not receive electrical stimulation. These results indicate that transfection of immune cells residing in muscle is increased by electroporation.

Table 1

Spleen	Luc activity	Fold increase after EP
EP1	82	>10
EP2	83	>10
NoEP	7.5	

Example 7 - Transfection of Immune Cells Residing in Skeletal Muscle of Mice:

Eleven mice were intramuscularly injected into the quadriceps with 50 μg of Luc plasmid DNA dissolved in 50 μl of 150 mM sodium phosphate buffer pH 7.2. In six of the eleven mice, the injection was followed by electroporation.

After two days, the lymph nodes of the mice were removed and analyzed for luciferase activity. As shown in Figure 6, the luciferase activity in the lymph nodes of the mice that received electroporation exhibited significantly greater luciferase activity that in the

mice that did not receive electroporation. These results indicate that electrical stimulation increases the transfection of immune cells residing in the muscle.

Example 8 - Transfection of Immune Cells Residing in Skeletal Muscle of Rats:

Six rats were intramuscularly injected in both surgically exposed soleus muscles and EDL with 50 μg of Luc plasmid DNA dissolved in 50 μl of 150 mM sodium phosphate buffer pH 7.2. After the injection, electrodes were inserted near the injection site and electroporation given to both EDL and Soleus on the right side of the animal.

After seven days, the lymph nodes of the rats were removed and analyzed for luciferase activity. Referring to Figure 7, the luciferase activity in the lymph nodes draining the right-side muscles that received electroporation was substantially higher than the activity of the lymph nodes draining the muscles on the left side that did not receive electroporation. These results indicate that electrical stimulation increases the transfection of immune cells residing in the muscle at the time of electroporation. The cells travel to the lymphoid tissue, where they could play a role in inducing an immune response.

Example 9 - Use of a Local Anaesthetic During Genetic Immunization:

Thirty-four mice were separated into groups of five to seven mice. Each mouse was intramuscularly injected in the quadriceps as follows. Group 1, saline +EP; group 2, *mpb70* no EP; group 3 *mpb70* and EP; group 4 *mpb70* + Marcain but no EP; group 5 *mpb70* Marcain and EP. Final concentration in the solution containing DNA was 1 μg/μl dissolved in 0.9% NaCl and group 4 & 5 also received 2.5 mg/ml Marcain in the DNA solution. Both muscles in each animal were injected with 50 μl of one

of these solutions.

The electrical stimulation was delivered shortly after injection to the muscles of selected mice and given near the site of injection. The sera were collected from the mice at four and eight weeks. A boost injection (50 μg of *mpb70*) was given after 8 week. A final ELISA was done on serum collected after 9 weeks. Referring to Figures 8-10, ELISA analysis of the sera revealed no significant differences between the genetically stimulated immune response in the animals that received Marcain and those that did not. Similar results are obtained with the 85B construct. These results were unexpected because Marcain is known to kill muscle cells.

Example 10 - Use of a Local Anaesthetic During Muscle Transfection/Electroporation:

Mice were divided into two groups: group 1 consisted of five mice, group 2 had six mice. Each mouse was injected with 50 μl with 25 μg CMV Luc plasmid DNA dissolved in 0.9% NaCl in each left quadriseps muscle. The right muscle received the same amount of DNA but mixed with Marcain to a final concentration of 0.5 μg/μl DNA and 2.5 mg/ml Marcain. All muscles in mice in group 1 were not electroporated. All muscles in group 2 received electroporation.

After five days, the animals were sacrificed and the quadriceps removed. The muscle were analyzed for luciferase activity. Figure 11 shows that animals that were injected with either CMV Luc plasmid DNA and or CMV Luc plasmid DNA and Marcain exhibited transfection at a high rate after the electroporation treatment. These data demonstrate that electroporation performed with or without an anaesthetic results in the same level of transfection.

Example 11 - Protein Immunization with Electroporation:

Six groups of mice were selected for use in the following protocol. Initial immunizations took place on day 0. Each mouse in the first group received an intramuscular injection of a saline solution followed by electroporation. A second group of three mice received an injection of 25 μg 85B protein and electroporation. Another group of three mice were injected with 25 μg 85B protein without electroporation. A group of five mice received 100 μg of *85b* DNA in solution without electroporation. Another group of five mice received 10 μg of *85b* DNA and electroporation. Finally, a third group of five mice received 100 μg of *85b* DNA and electroporation.

Eight weeks after the initial immunization, all animals were given a second immunization in which each animal in all six groups received an intramuscular injection of a mixture of 25 μg of *85b* DNA and 1 μg Luc DNA, followed by electroporation. Five days later, the muscles were removed and assayed for luciferase activity. In animals in which a strong cellular immune response was induced by the first immunization, one might expect to see a reduced luciferase activity compared to those animals without a good induction of the cellular immune response. Without being bound by any particular theory, one might expect to see this in two situations: when the cellular immune response is lacking or repressed, or when the humoral branch of the immune system is activated, such that the second immunization with DNA primes the existing humoral response rather than a non-stimulated cellular response.

The results of this assay are shown in Figure 12. The treatment each mouse received on day 0 is written on top of each bar. For example, "NaCl-85B" on top of the bar means the group received saline at day 0 and the mixture of *85b* and luciferase at week 8.

DNA without electrical stimulation did not have

much effect compared to saline. Both doses of DNA with electrical stimulation had an effect, shown by the low luciferase activity. Without being bound by any particular theory, it appears that transfection at day 0 with electroporation caused a cellular immune response that was rapidly mobilized and killed 85B/Luc-expressing fibers five days after the boost injection at week 8.

However, with the protein, something else occurred as shown by the large increase in luciferase activity. These results could be caused by a type of immune tolerance or deviation. That is, the immune reaction has changed to a humoral type that was enhanced by the boost injection. This humoral-type immune response did not result in killing of the transfected muscle fibers.

Example 12 - Protein Immunization Followed by DNA Booster:

Eight mice received NaCl and EP (control group), nine mice received protein 85B (group, 85B + *85b*), five mice got protein MPB70 (group, MPB70+*mpb 70*) at day 0. The protein was given as an intra muscular injection of 20 μg purified 85B or MPB70 protein and electrical stimulated (right muscle only).

Eight weeks after the initial immunization, the animals were given a booster injection with DNA (35 μg in 50 μl 0.9 % NaCl) encoding for the corresponding protein antigen given in the first injection. The control group was split in two: four mice received *mpb70* (group NaCl + *mpb 70*) and the other four received *85b* group (NaCl ±*85b*). The subsequent antibody response was measured five weeks later with ELISA. If the humoral response was stimulated/primed by the protein injection, one would expect to see a stronger increase in IgG1 antibodies after immunization with DNA.

Referring to Figure 13, an elevated level of IgG1 was detected in the mice that received the initial protein, either 85B or MPB70 vaccination indicating that

a humoral response was induced in these mice compared with mice that only received DNA. To demonstrate specificity of the assay, the ELISA was also done on serum from animals immunized with a different construct, hence the 85B ELISA was done on serum from animals that were previously immunized with the *mpb70* plasmid (to serve as a negative control). No cross-reaction was observed.

Example 13 - Low Voltage DNA Immunization:

We have tested relatively low (less than 100 V/cm) electric field strength, which could be used to avoid stimulation of and damage to surrounding tissue. We used a low voltage, which we did not expect would have much effect on transfection of muscle fibers. The low voltage, however, still had a significant effect on immunization.

Four groups of mice were selected for the following protocol. Each mouse was intramuscularly injected in the quadriceps as follows. The first group of six mice were injected with 0.9% saline and exposed to electroporation. Another group of six mice were injected with 100 μg *mpb70* plasmid DNA dissolved in 0.9% NaCl and received no electroporation. A third group consisting of seven mice were injected with 100 μg *mpb70* plasmid DNA dissolved in 0.9% NaCl and received electroporation at standard field strengths. A final group of seven mice were injected with 100 μg *mpb70* plasmid DNA dissolved in 0.9% NaCl and received electroporation at lower field strengths.

The electrical stimulation was delivered shortly after injection and given near the site of injection. Each mouse was electrically stimulated with 8 trains of 1000 pulses at 1000 Hz. Each pulse lasted for 200 μs positive and 200 μs negative for a total pulse duration of total 400 μs. The electrical field strength varied with the change in resistance in the tissue of each

animal, but the field strength for the standard voltage level was in the range of approximately 50-70 V over approximately 3-4 mm, or from about 125 to about 233 V/cm. The electric field strength was from about 10 V/cm to about 25 V/cm at the low voltage electroporation. This low voltage stimulation caused strong muscle contraction. Each train was delivered at two second intervals with each train lasting one second.

After four and eight weeks, the sera of the mice was collected and ELISA performed on sera. Figure 14 shows the results of the eight week ELISA. The results were similar at four weeks, but are not shown. This experiment was also done with a different antigen 85B with similar results.

The eight week results show that low voltage stimulation enhances immune response compared to naked DNA injection. This enhanced immune response could be due to the induced muscle activity or by transfecting cells other than muscle cells such as immune cells residing within the muscle.

Example 14 - Increased Numbers of CD8- and CD4-Positive Cells After Immunization Using EP:

Twelve Balb/C mice were separated into four groups. Three mice received *85a* and EP, three received *85a* without EP, three mice received a plasmid encoding β-galactosidase (*β-gal*, see previous Examples for details about construct) with EP, and three mice received *β-gal* without EP. Fourteen days later the spleens were removed from the animals. Cells were isolated and treated according to standard ELISPOT procedures. *See* Schneider et al, *Nature Medicine* 4:397-402 (1998). Briefly, 1, 0.5 and 0.25 million spleenocytes from each animal were placed in duplicates of antibody-coated wells (anti-mouse INF-gamma mAb R4-6A2, hybridoma from European Collection of Animal Cell Cultures). Peptides (concentration 1 μg/ml) were added to each test well.

Control wells received irrelevant peptide. After incubation overnight, plates were washed and incubated for 3 hours with a solution of 1 μg/ml biotinylated anti-mouse INF-gamma mAb XMG1.2 (Pharmingen, CA), washed, and incubated for 2 hr with 50 μl of a 1 mg/ml solution of Streptavidin-Alkaline-Phosphatase polymer (Sigma) at RT. Spots were developed by adding 50 μl of an alkaline phosphatese conjugated substrate solution (Biorad, Hercules, CA) and reactions were stopped by washing with water. Spots were counted electronically.

The peptides used to stimulate spleenocytes from *85a* immunized animals were: P-11, an epitope from 85A that specifically binds to MHC class I and thereby stimulates CD8 positive cells (Figure 15 A); P-15, an epitope from 85A that specifically binds to MHC class II and thereby stimulates CD4 positive cells (Figure 15 B). See Denis et al., *Infect. Immun.* 66:1527-1533 (1998) for details about the peptides.

The peptide used to stimulate spleenocytes from *β-Gal* immunized animals were AA 876-884 from *E. Coli* beta-galactosidase, this peptide specifically binds to MHC class I and thereby stimulates CD8-positive cells. See Figure 15 C.

Results shown in Figure 15 demonstrate an increased number of both CD4 and CD8 positive T-cells when immunization is done in combination with EP. Hence, the cellular branch of the immune system is stimulated.

A high number of CD8- and CD4-positive T-cells is often associated with good protection against many serious infectious diseases in vaccinated humans. It is also believed to be important in protection and the treatment of cancer.

Example 15 - Demonstration of transfection of non-muscle cells

Male Wistar rat EDL and soleus muscles were injected with 0.4 μg/μl of DNA plasmid containing the

GEP gene in 50 µl 0.9% NaCl. Shortly after injection the muscles were electrically stimulated with 10 trains of pulses. Each train were built up of 1000 symmetrical bipolar square pulses lasting 200 µs in each polar direction and with an amplitude of 150 V/cm p-p. Between each train there were a 1 second pause. Distance between the electrodes was between 2 and 4 mm.

One day after DNA transfection the muscles were surgically removed. After removal the muscles were stretched out and pinned down on a petridish with sylgard followed by incubation in a freshly made 2% paraformaldehyde solution. The muscles were then teased from tendon to tendon into 10-14 pieces in a 0.1 M phosphate-buffered saline solution (PBS).

Muscle bundles stained with the primary antibodies targeted against Thy-1 (0X7, undiluted supernatant), CD43 (W3/W13, undiluted supernatant), MHCII (0X6, undiluted supernatant) and CD45 (OX1, undiluted supernatant) were incubated for 30 minutes with 5% goat serum in PBS to block unspecific binding of the secondary antibody. This was followed by incubation with the undiluted primary antibody with 5% goat serum over night at 4°C. After washing 3 times with PBS the muscle bundles were incubated with a goat anti-mouse rhodamin conjugated secondary antibody (R-6393, 1:500 in PBS, Molecular Probes, Eugene, OR) at RT for 1 hr followed by washing 3 times with PBS.

Antibodies were used to stain cells expressing S-100 (Z0311, rabbit IgG, DAKO, Denmark) and vimentin (V6630, mouse IgG, Sigma-Aldrich, St. Louis, MO). Muscle bundles were permeablized with chilled methanol for 10 minutes at -20°C, followed by washing 3 times in PBS and 30 minutes incubation with 0.2% bovine albumin (A-7906, Sigma-Aldrich) for Z0311 and 5% goat serum for V6630. The primary antibody were diluted 1:400 in a PBS solution containing 0.2% bovine albumin for Z0311 and 5% goat serum for V6630, 0.3% Triton X-100 and 0.1%

NaAzide. After incubation of muscle bundles for 1 hr at RT with the primary antibody the bundles were washed 3 times with PBS. The bundles stained with Z0311 were then treated with the goat Cy3 conjugated anti-rabbit secondary antibody (1:500 in PBS, 111-166-003, Jackson ImmunoResearch Laboratories, West Grove, PA) and bundles stained with V6630 were treated with R-6393 (1:500 in PBS) for 1 hr at RT followed by washing in PBS 3 times. After staining the muscle pieces were squeezed flat between slide and coverslip. The pieces were then examined with a fluorescent microscope fitted with epifluorescent illumination suitable for studying GFP, rhodamin and Cy3.

Results:

One day after DNA transfection on average 100 non-muscle cells expressed GFP in the muscle. Some of these cells were positive for vimentin, S-100 or thy-1. The GFP expressing cells closely associated with MHCII positive cells. The correspondence between cells expressing GFP due to transfection by the method of the invention and those expressing S-100, invention and thy-1 can be seen from Figures 16-18.

Accordingly not only skeletal muscle cells but also a significant number of other cells were transfected. Cell morphology and immunostaining indicate that in addition to muscle cells there are connective tissue cells and adipose cell. This shows that the described DNA transfection method is not restricted to transfection of large elongated muscle cells, but also other cells, e.g. smaller cells with dendrites and variable size and morphology. Some of the cells transfected did not stain positive for conventional markers of connective tissue cells. This indicates that other cells such as dendritic cells and macrophages also can be transfected by the method of the invention. These cells may include cells which do not stay in the

muscle after transfection but wander to the draining lymph node and there induce the immune reaction.

Example 17 - Vaccination applied in large animals

Goats were immunized with DNA as follows: xylazin was given (intra muscularly) as sedative and analgesic (0.2 mg/kg), and further the goats were given ketamine (5 mg/kg body weight) or lidocain (50 mg) as anaesthetic intra muscularly about 5-15 minutes before treatment. The animals were left in their stable until steady sleep. After DNA injection with electroporation, the goats were given the anti-sedative atipamezol (0.05mg/kg body weight) (α_2-antagonist) to avoid stomach trouble. DNA was given as two injections of 100 μl DNA solution (a mixture of *mpb70* and *85b* both in the concentration of 0.5μg/μl saline) intra muscularly in the right *Gastroc nemicus* (medial-lateral). Blood samples were taken every fortnight and assayed by ELISA (see example 4).

Results

Sedative and local anaesthetics were sufficient to avoid pain for the animals. Further, the goats recovered fast and stiffness was gone one day after the stimulation. We observed little or slight contractions of the muscle during the procedure when local anaesthetics were used.

Measurement of antibodies in serum from the electroporated goats shows a higher antibody titre than non-electroporated goats at 4 weeks after treatment. Results are shown in Figure 19 which shows graphs of serum IgG levels against 85B and MPB70 over a 20 week period after injection (bold symbols) or after injection with electroporation (fine symbols). Electroporated goats show an elevated serum IgG level after 4 weeks in both experiments.

The results show that it is possible to vaccinate goats with DNA in combination with electroporation by

just using sedatives and local anaesthetics. In addition, DNA vaccination in combination with electroporation shows a higher immune response than non-electroporated animals even in the presence of the local anesthetics ketamine lidocain.

Claims

1. A method of eliciting an immune system response in a mammal, which method comprises:

injecting a first antigen into a first injection site in skeletal muscle in said mammal;

optionally injecting a polynucleotide functionally encoding a second antigen into a second injection site in skeletal muscle in said mammal;

positioning electrodes in said skeletal muscle such that current travelling between said electrodes passes through said first and/or second injection site; and

electrically stimulating said skeletal muscle with an electrical current between said electrodes having a field strength in said skeletal muscle of from 10 to 300 V/cm whereby to assist in cellular uptake of said first antigen and/or said polynucleotide.

2. A method as claimed in claim 1 wherein said first antigen comprises a protein or protein fragment.

3. A method as claimed in claim 2 wherein said first antigen and said polynucleotide are injected into skeletal muscle of said mammal.

4. A method as claimed in claim 3 wherein said first antigen is injected before said polynucleotide.

5. A method as claimed in either of claims 3 and 4 wherein electrical stimulation is used to assist in cellular uptake of said first antigen and of said polynucleotide.

6. A method as claimed in any one of claims 3 to 5 wherein said first antigen and said second antigen share at least one common antigenic epitope.

7. A method as claimed in any one of claims 1 to 6 wherein said polynucleotide is DNA.

8. A method as claimed in claim 7 wherein said polynucleotide is in a plasmid.

9. A method as claimed in any one of claims 1 to 8 wherein said first antigen and/or said polynucleotide and a local anaesthetic are injected simultaneously or sequentially.

10. A method as claimed in any one of claims 1 to 9 wherein said field strength is from 12 to 233 V/cm.

11. The use of an antigen and/or a polynucleotide functionally encoding an antigen for the manufacture of pharmaceutical composition for use in a method of eliciting an immune response as claimed in any one of claims 1 to 10.

12. The use of an antigenic substance and/or a polynucleotide functionally encoding an antigen for the manufacture of a pharmaceutical composition for use in a method of introducing said antigenic substance and/or said polynucleotide into non-muscle cells which comprises injecting said composition into an injection site in skeletal muscle of a mammal and electroporating cells at said injection site.

13. The use of a polynucleotide functionally encoding an antigen for the manufacture of a pharmaceutical composition for use in a method of treatment of a mammal to induce a cellular immune response, which method comprises injecting said composition into an injection site in skeletal muscle of said mammal and electroporating cells around said injection site.

14. The use of an antigen for the manufacture of a pharmaceutical composition for use in a method of treatment of a mammal to induce a humoral immune response, which method comprises injecting said composition into an injection site in skeletal muscle of said mammal and electroporating cells around said injection site.

15. Use as claimed in any one of claims 11 to 14 of a first antigen and/or a polynucleotide as defined in any one of claims 2 to 8.

16. A pharmaceutical composition antigen for use in a method of eliciting an immune response according to the invention, said composition comprising an antigen and/or a polynucleotide functionally encoding an antigen, together with a physiologically tolerable carrier or excipient.

17. A pharmaceutical composition comprising a proteinaceous antigen and cell-free polynucleotide functionally encoding a protein antigen, optionally together with a physiologically acceptable carrier or excipient.

18. A composition as claimed in either of claims 16 and 17 further comprising a local anaesthetic.

19. An antigen and/or a polynucleotide functionally encoding an antigen for use in a method of eliciting an immune response according to any of claims 1 to 10.

20. A kit comprising: a first pharmaceutical composition comprising a proteinaceous antigen optionally together with a physiologically acceptable carrier or excipient; and a second pharmaceutical composition comprising cell-free polynucleotide

functionally encoding a protein antigen optionally together with a physiologically acceptable carrier or excipient.

5

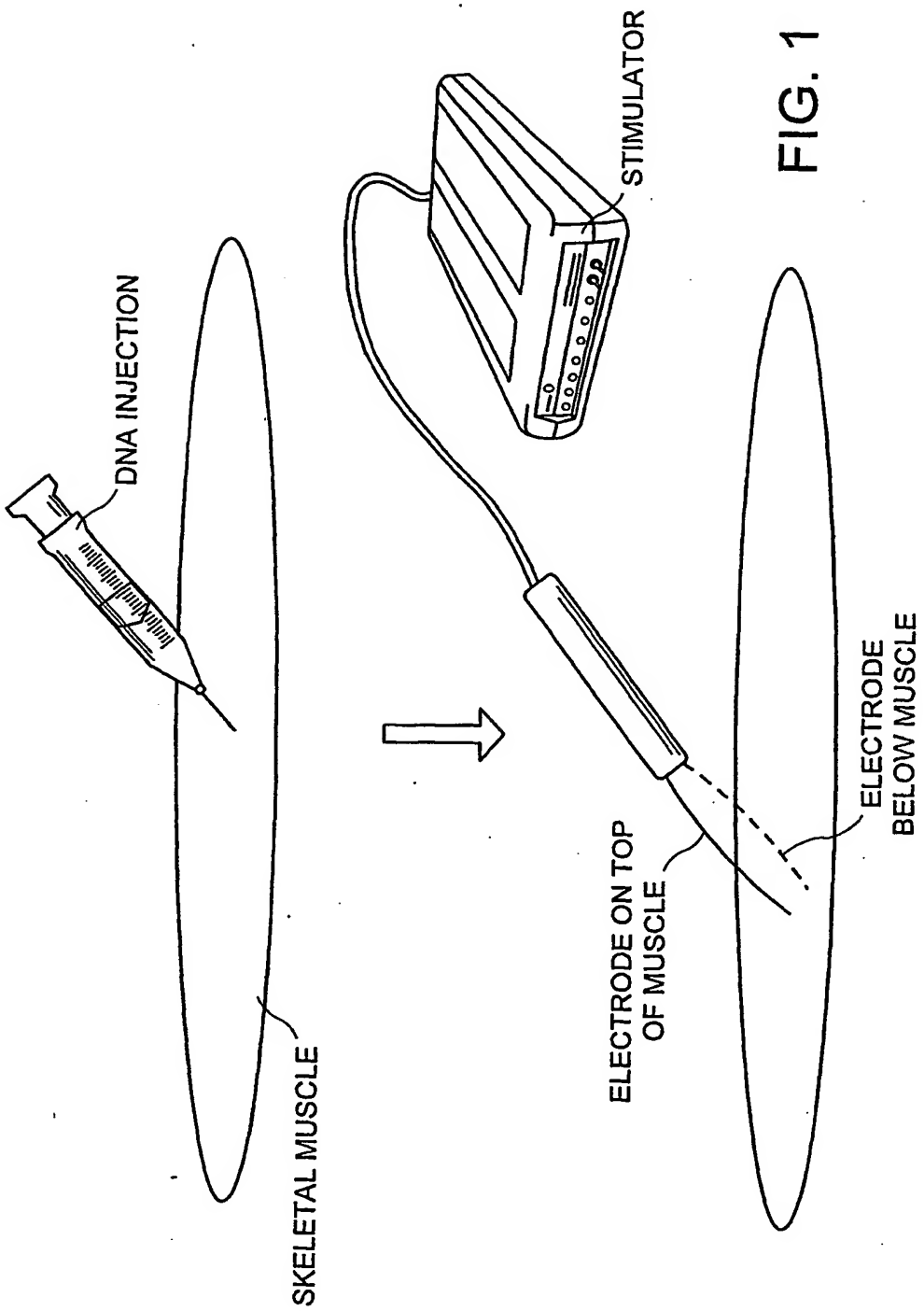

DNA INJECTION
SKELETAL MUSCLE
STIMULATOR
ELECTRODE ON TOP OF MUSCLE
ELECTRODE BELOW MUSCLE

FIG. 1

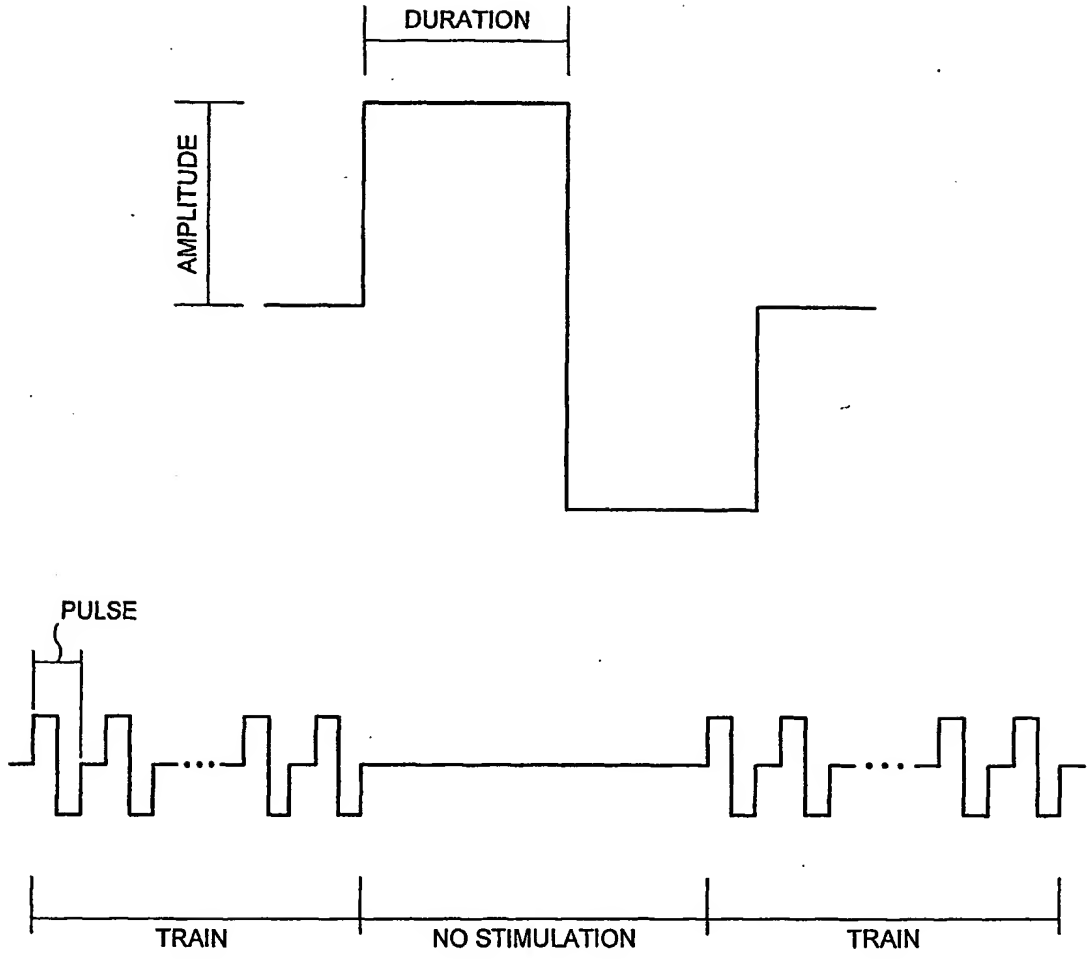


FIG. 2

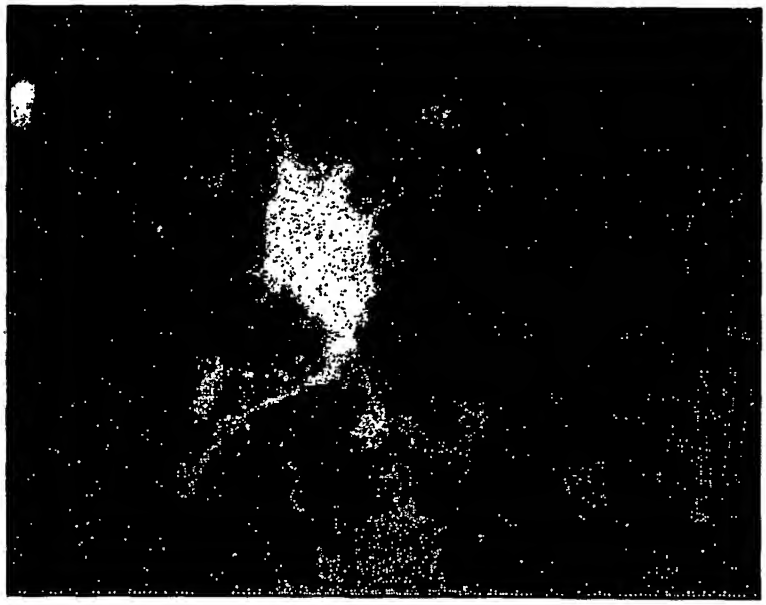


FIG. 3A

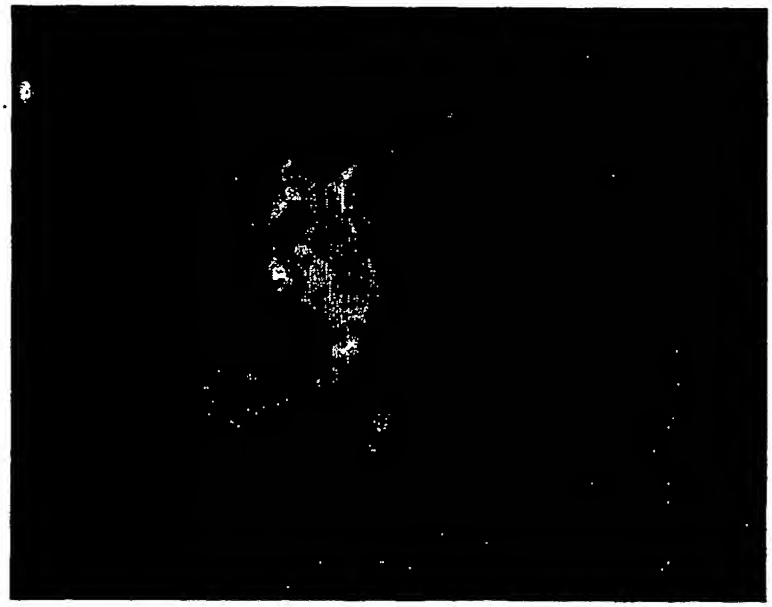


FIG. 3B

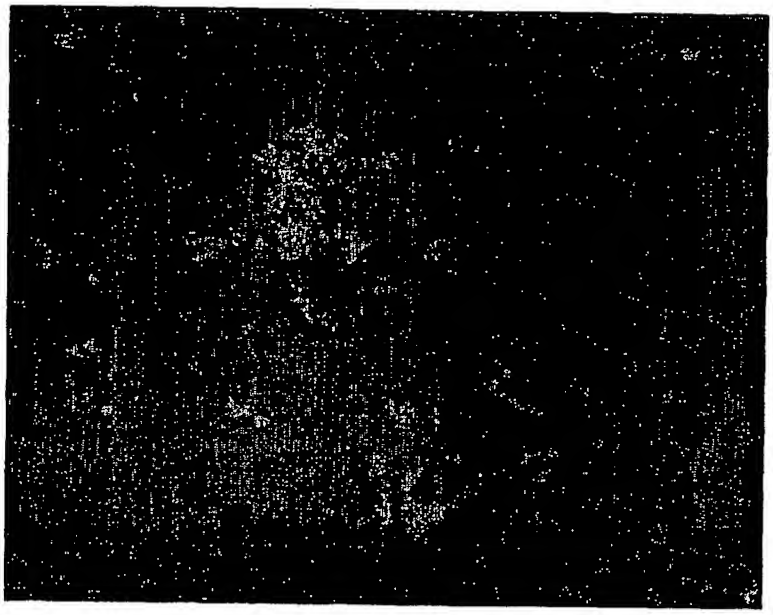


FIG. 3C

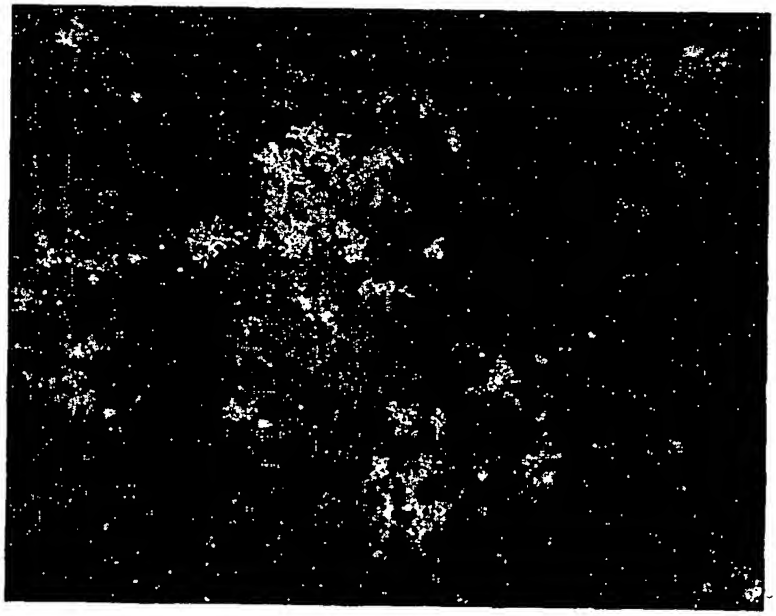


FIG. 3D

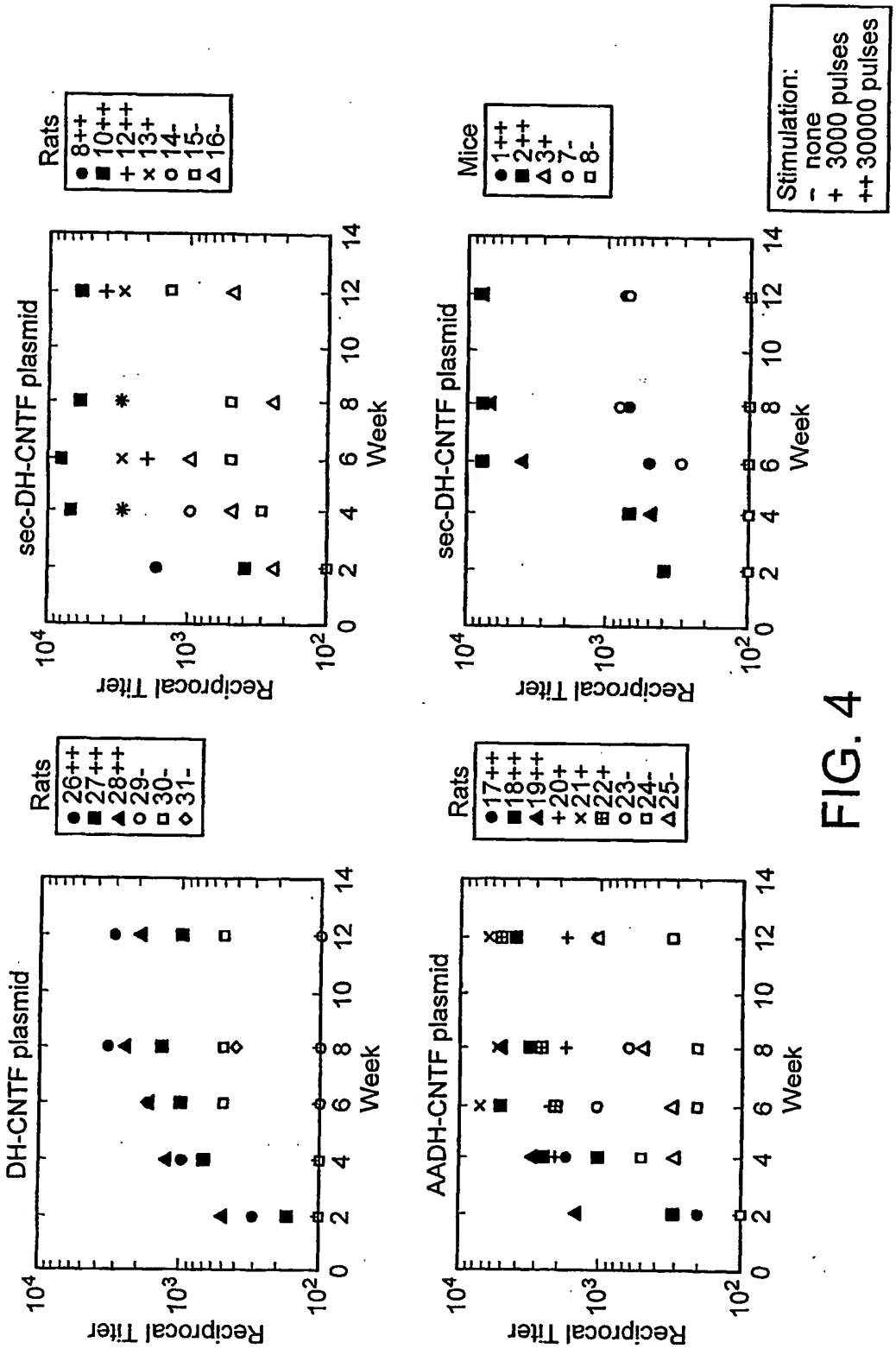

Rats
8++
10++
12++
13+
14-
15-
16-
sec-DH-CNTF plasmid
Reciprocal Titer
Week
Mice
1++
2++
3+
7-
8-
Stimulation:
- none
+ 3000 pulses
++ 30000 pulses
Rats
26++
27++
28++
29-
30-
31-
DH-CNTF plasmid
Rats
17++
18++
19++
20+
21+
22+
23-
24-
25-
AADH-CNTF plasmid

FIG. 4

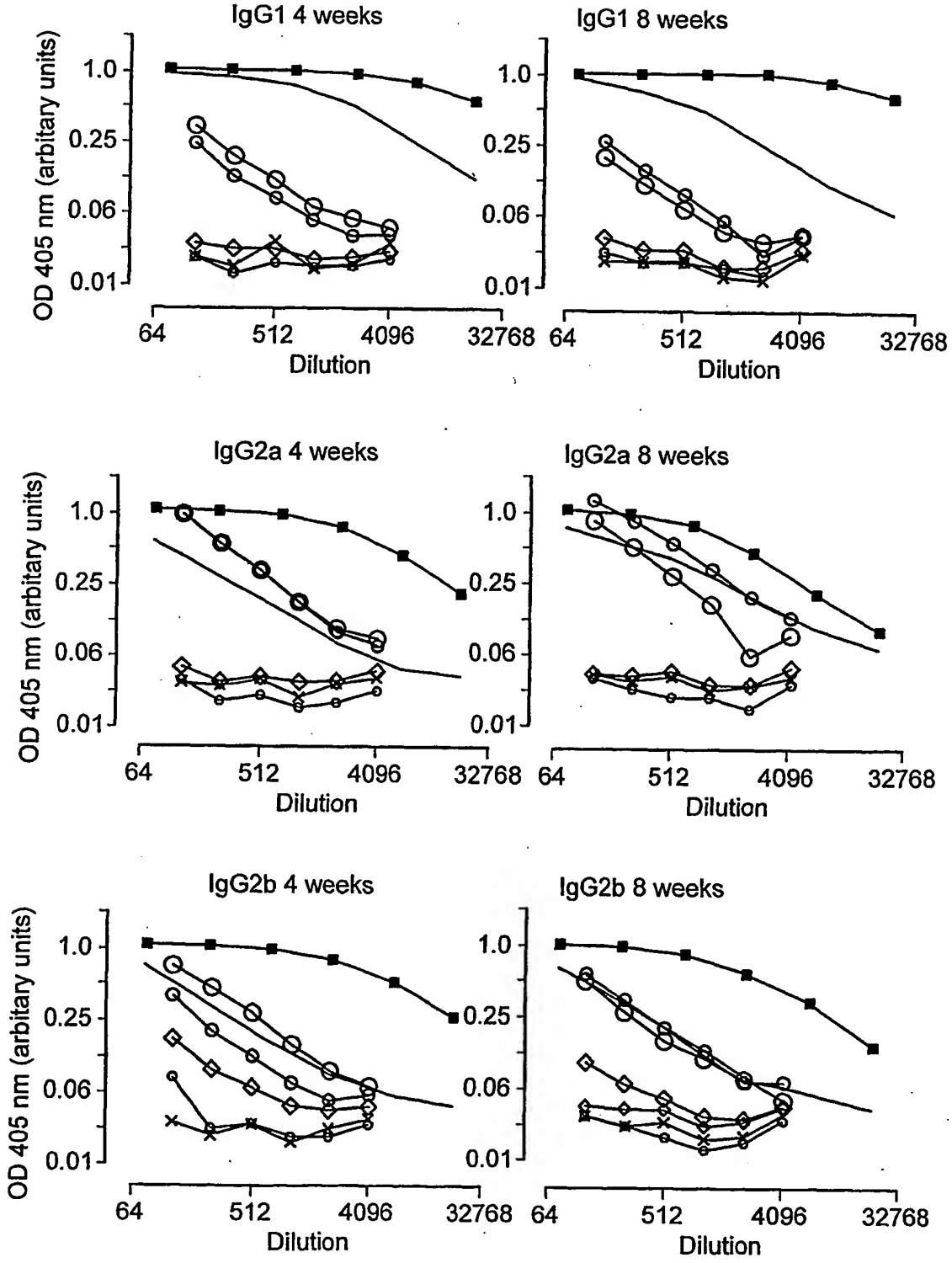


FIG. 5A Specific Antibodies against MPB70

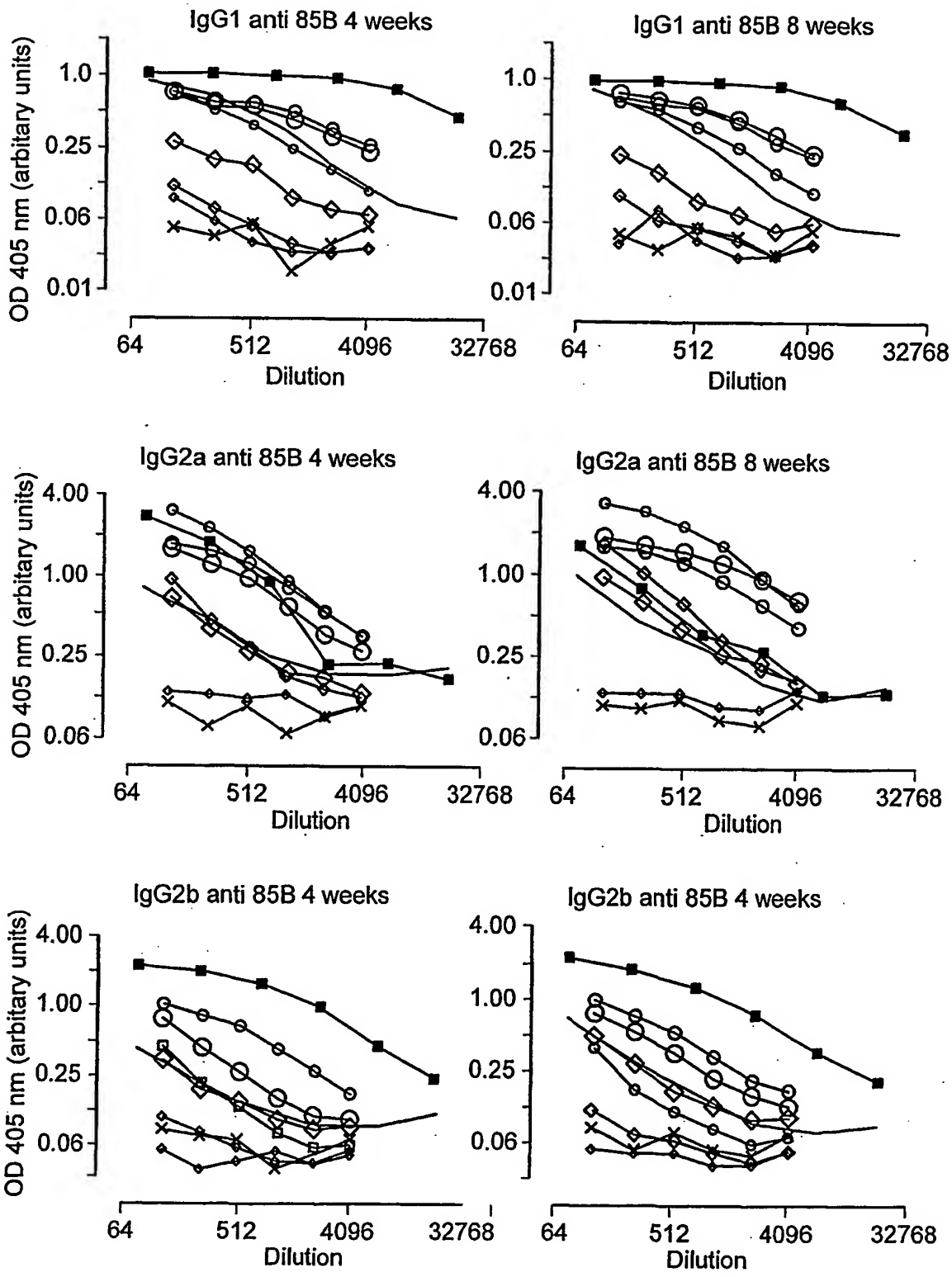


FIG. 5B Specific Antibodies against 85B

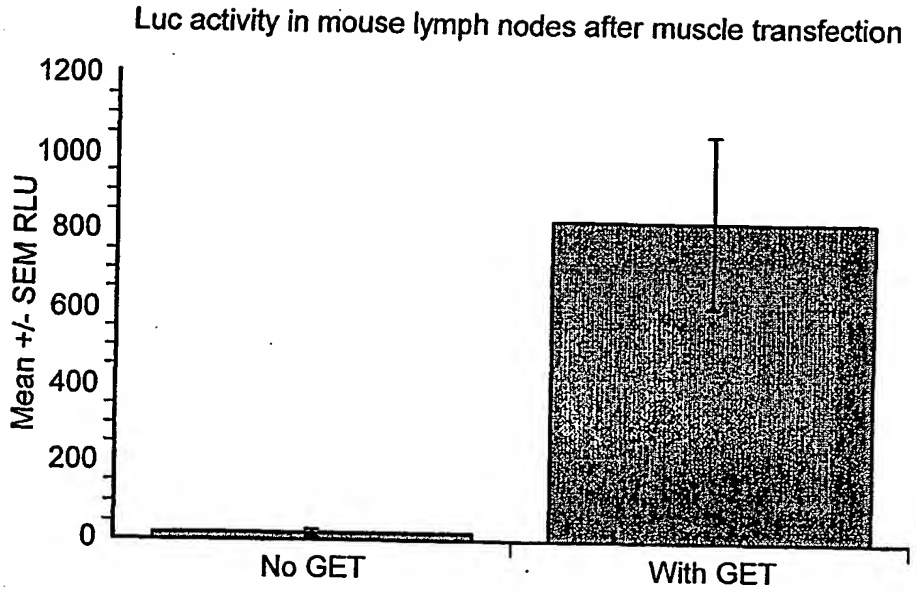

Luc activity in mouse lymph nodes after muscle transfection
Mean +/- SEM RLU
1200
1000
800
600
400
200
0
No GET
With GET

FIG. 6

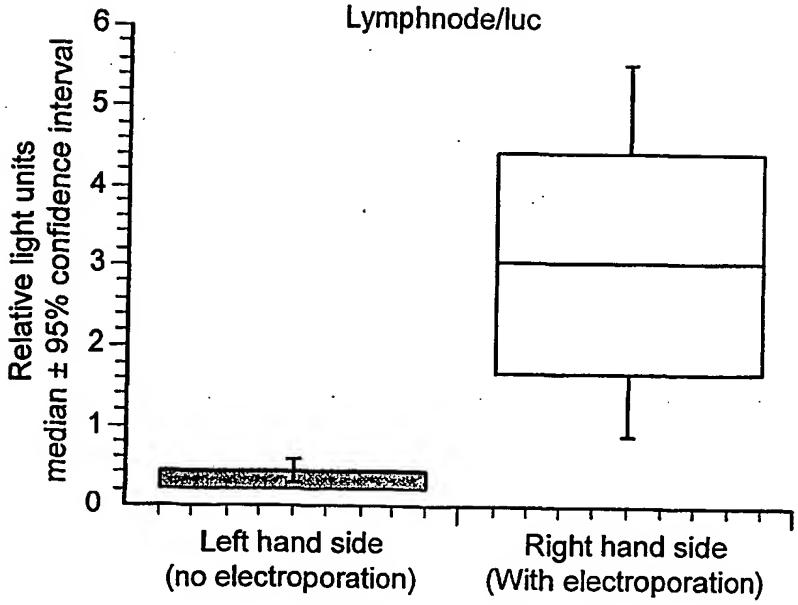

Lymphnode/luc
Relative light units
median ± 95% confidence interval
6
5
4
3
2
1
0
Left hand side
(no electroporation)
Right hand side
(With electroporation)

FIG. 7

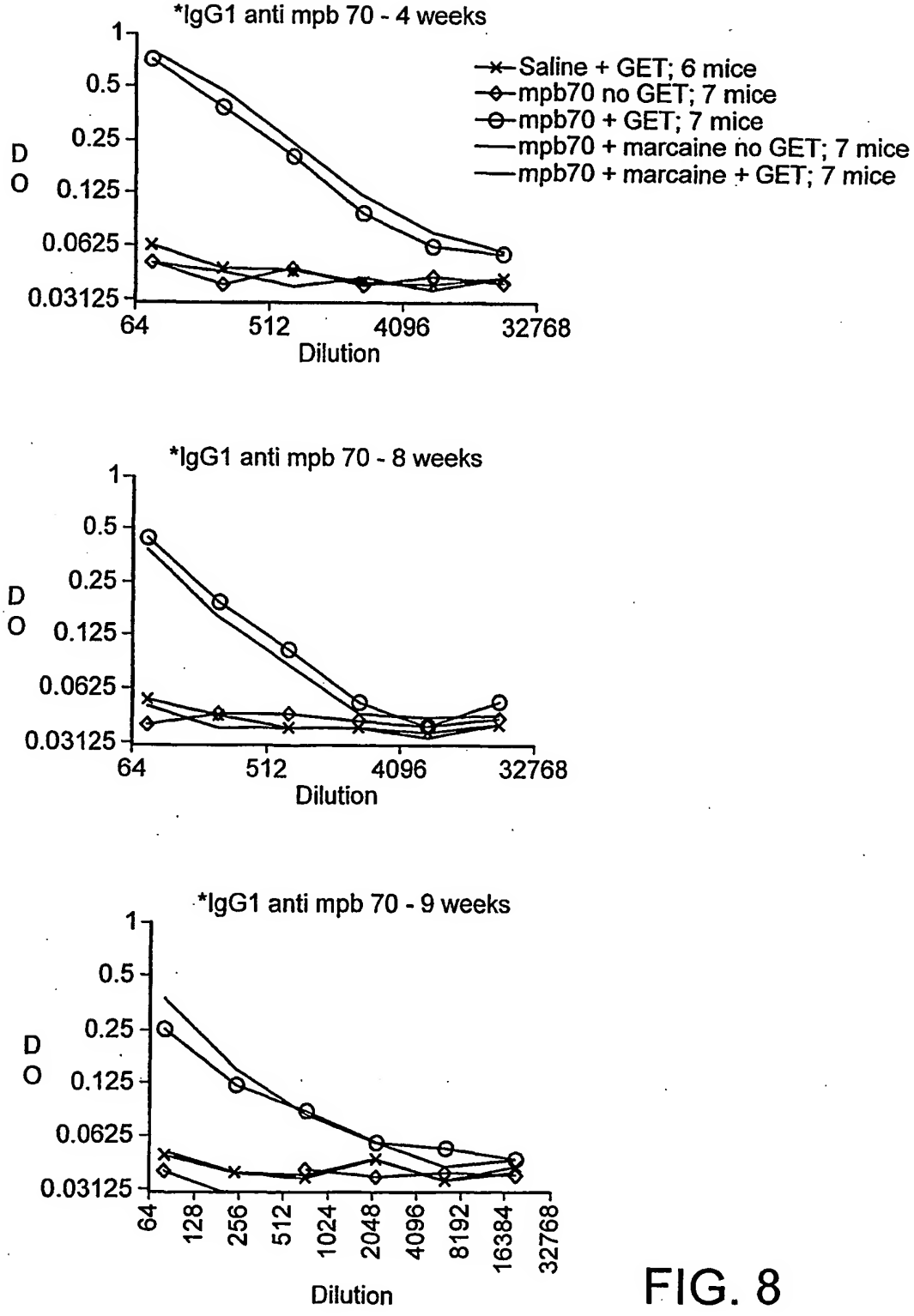
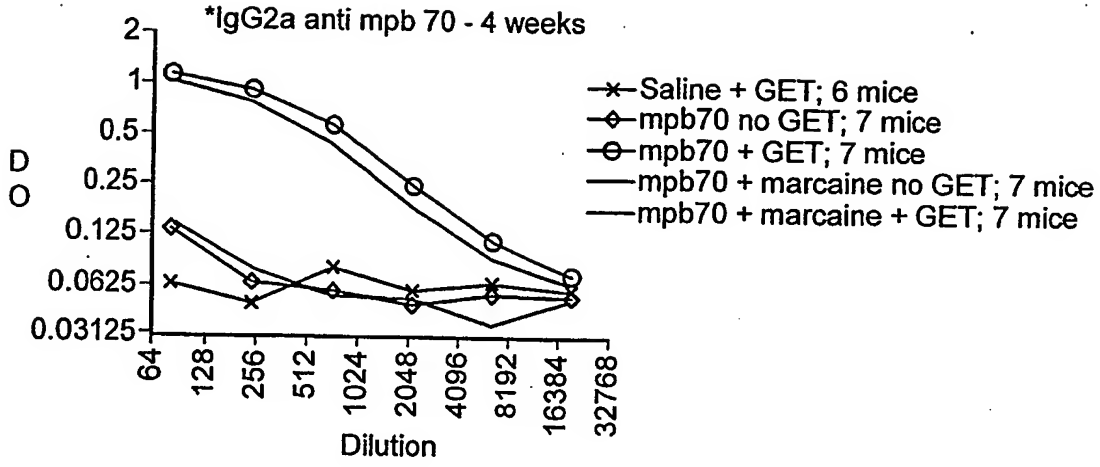
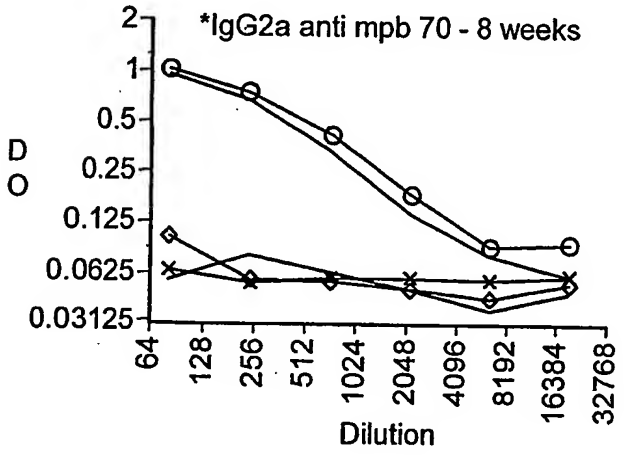

*IgG1 anti mpb 70 - 4 weeks
Saline + GET; 6 mice
mpb70 no GET; 7 mice
mpb70 + GET; 7 mice
mpb70 + marcaine no GET; 7 mice
mpb70 + marcaine + GET; 7 mice
D O
1
0.5
0.25
0.125
0.0625
0.03125
64
512
4096
32768
Dilution
*IgG1 anti mpb 70 - 8 weeks
D O
1
0.5
0.25
0.125
0.0625
0.03125
64
512
4096
32768
Dilution
*IgG1 anti mpb 70 - 9 weeks
D O
1
0.5
0.25
0.125
0.0625
0.03125
64
128
256
512
1024
2048
4096
8192
16384
32768
Dilution

FIG. 8


*IgG2a anti mpb 70 - 4 weeks
DO
2
1
0.5
0.25
0.125
0.0625
0.03125
64
128
256
512
1024
2048
4096
8192
16384
32768
Dilution
Saline + GET; 6 mice
mpb70 no GET; 7 mice
mpb70 + GET; 7 mice
mpb70 + marcaine no GET; 7 mice
mpb70 + marcaine + GET; 7 mice


*IgG2a anti mpb 70 - 8 weeks
DO
2
1
0.5
0.25
0.125
0.0625
0.03125
64
128
256
512
1024
2048
4096
8192
16384
32768
Dilution

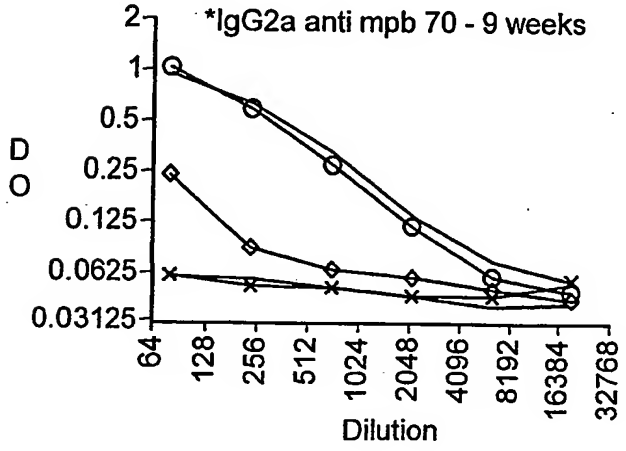

*IgG2a anti mpb 70 - 9 weeks
DO
2
1
0.5
0.25
0.125
0.0625
0.03125
64
128
256
512
1024
2048
4096
8192
16384
32768
Dilution

FIG. 9

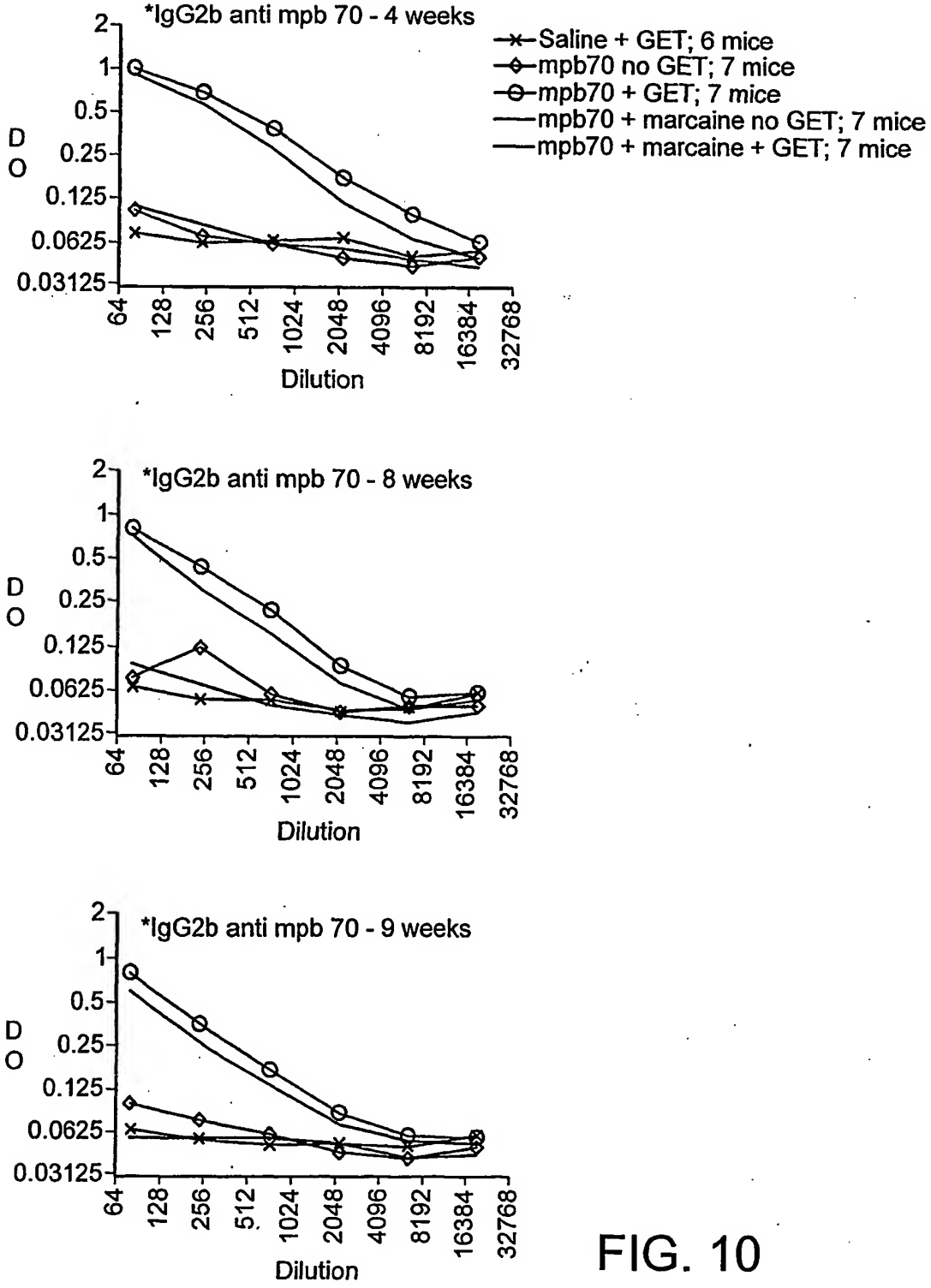

*IgG2b anti mpb 70 - 4 weeks
Saline + GET; 6 mice
mpb70 no GET; 7 mice
mpb70 + GET; 7 mice
mpb70 + marcaine no GET; 7 mice
mpb70 + marcaine + GET; 7 mice
D O
2
1
0.5
0.25
0.125
0.0625
0.03125
64
128
256
512
1024
2048
4096
8192
16384
32768
Dilution
*IgG2b anti mpb 70 - 8 weeks
*IgG2b anti mpb 70 - 9 weeks

FIG. 10

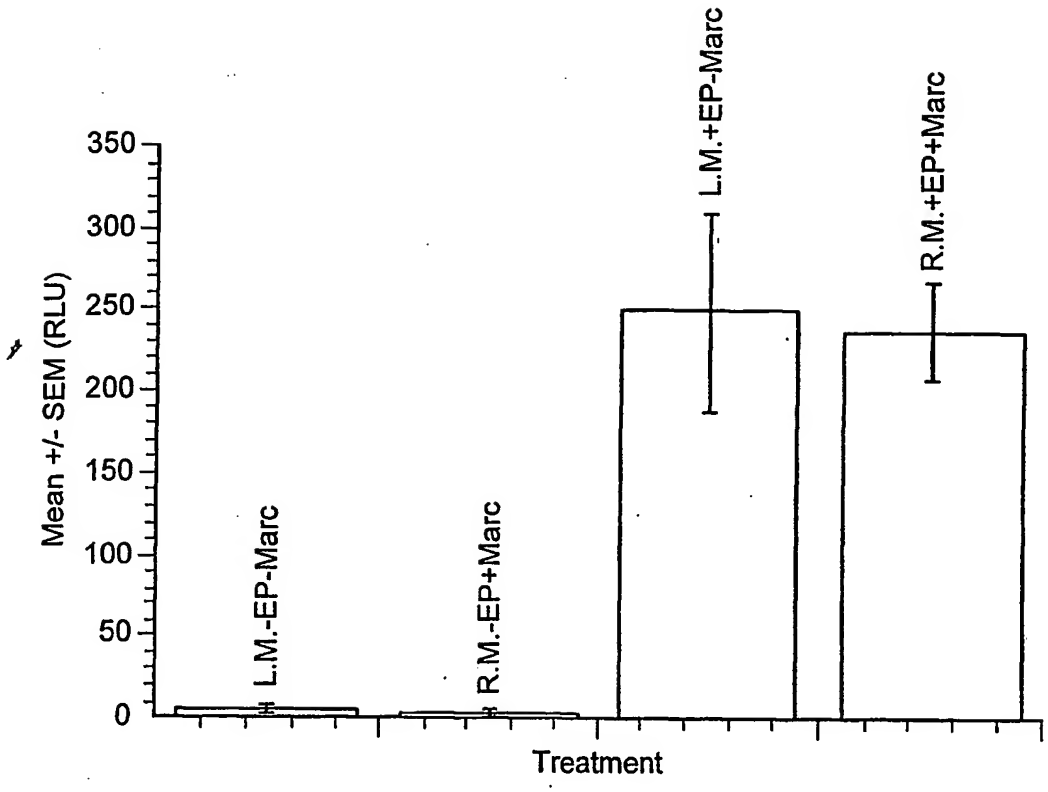

Mean +/- SEM (RLU)
350
300
250
200
150
100
50
0
L.M.-EP-Marc
R.M.-EP+Marc
L.M.+EP-Marc
R.M.+EP+Marc
Treatment

FIG. 11

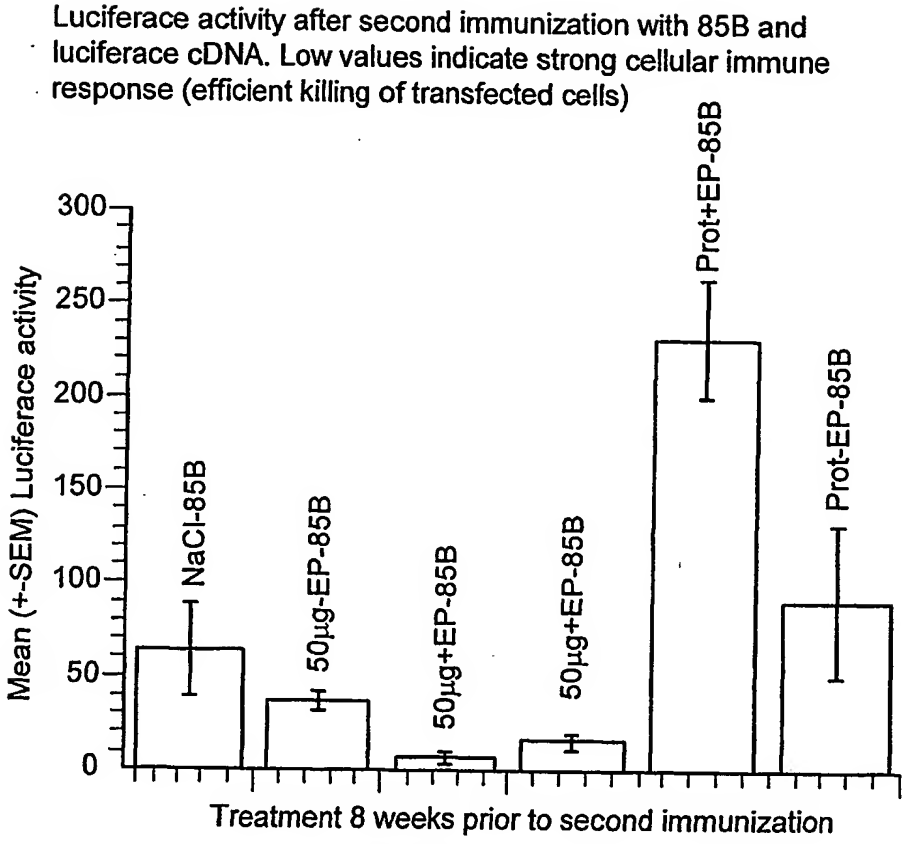

Luciferace activity after second immunization with 85B and luciferace cDNA. Low values indicate strong cellular immune response (efficient killing of transfected cells)
Mean (+-SEM) Luciferace activity
300
250
200
150
100
50
0
NaCl-85B
50µg-EP-85B
50µg+EP-85B
50µg+EP-85B
Prot+EP-85B
Prot-EP-85B
Treatment 8 weeks prior to second immunization

FIG. 12

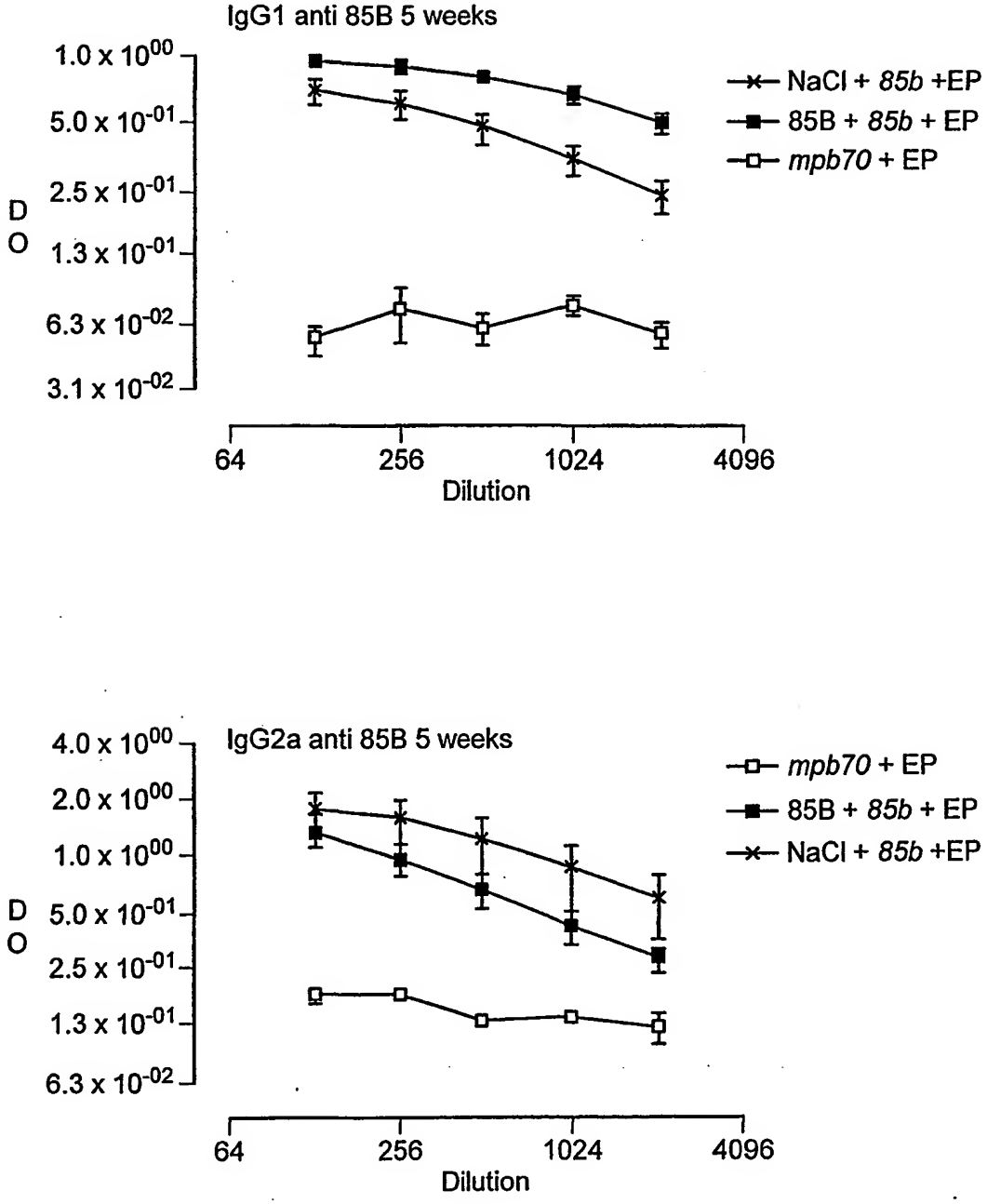
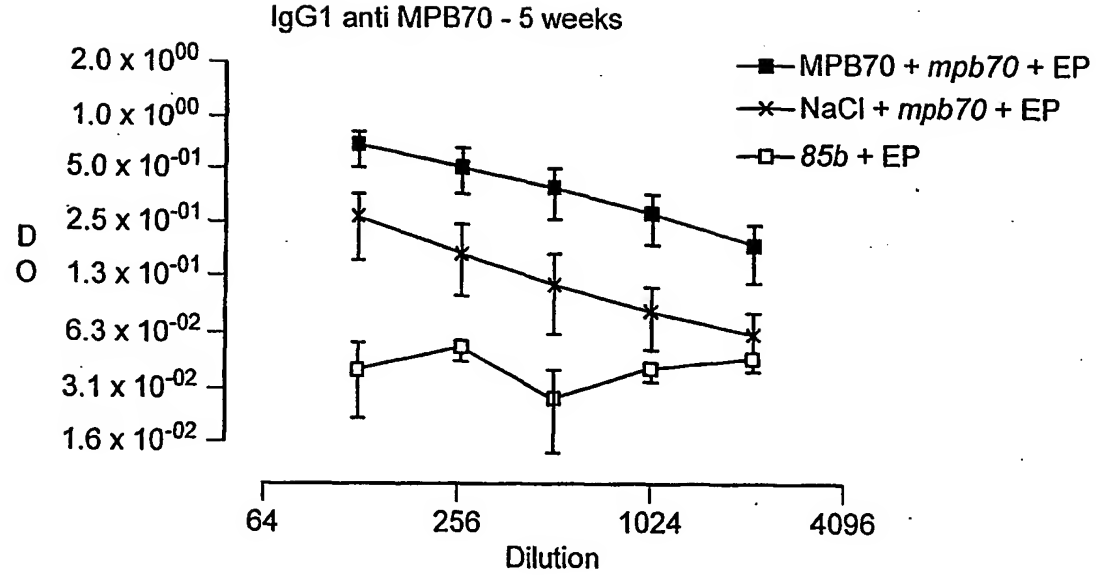

IgG1 anti 85B 5 weeks
1.0 x 10^00
5.0 x 10^-01
2.5 x 10^-01
1.3 x 10^-01
6.3 x 10^-02
3.1 x 10^-02
D
O
64
256
1024
4096
Dilution
NaCl + 85b +EP
85B + 85b + EP
mpb70 + EP
IgG2a anti 85B 5 weeks
4.0 x 10^00
2.0 x 10^00
1.0 x 10^00
5.0 x 10^-01
2.5 x 10^-01
1.3 x 10^-01
6.3 x 10^-02
D
O
64
256
1024
4096
Dilution
mpb70 + EP
85B + 85b + EP
NaCl + 85b +EP

FIG. 13A


IgG1 anti MPB70 - 5 weeks
2.0 x 10^00
1.0 x 10^00
5.0 x 10^-01
2.5 x 10^-01
1.3 x 10^-01
6.3 x 10^-02
3.1 x 10^-02
1.6 x 10^-02
D
O
MPB70 + mpb70 + EP
NaCl + mpb70 + EP
85b + EP
64
256
1024
4096
Dilution

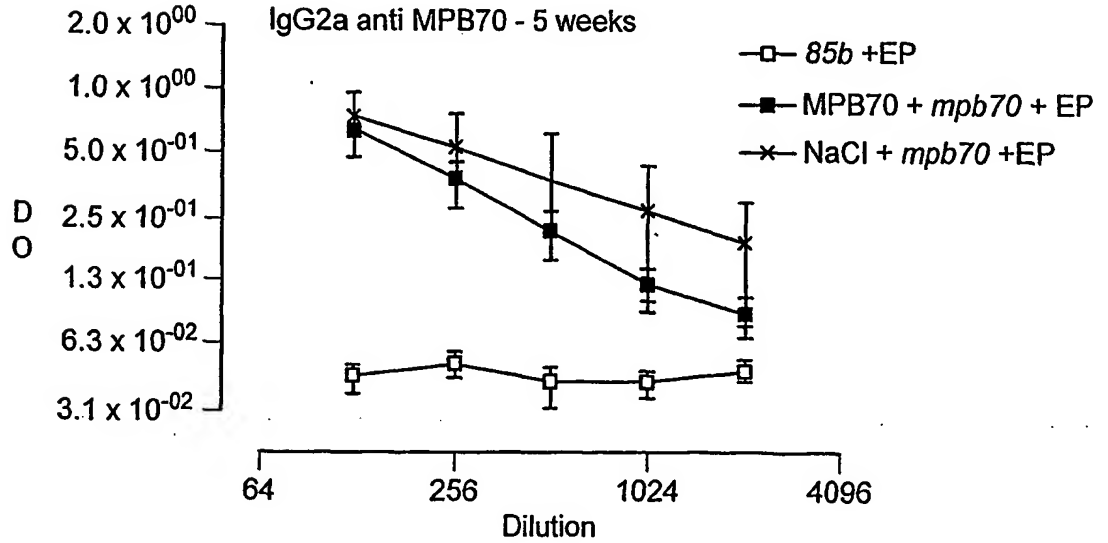
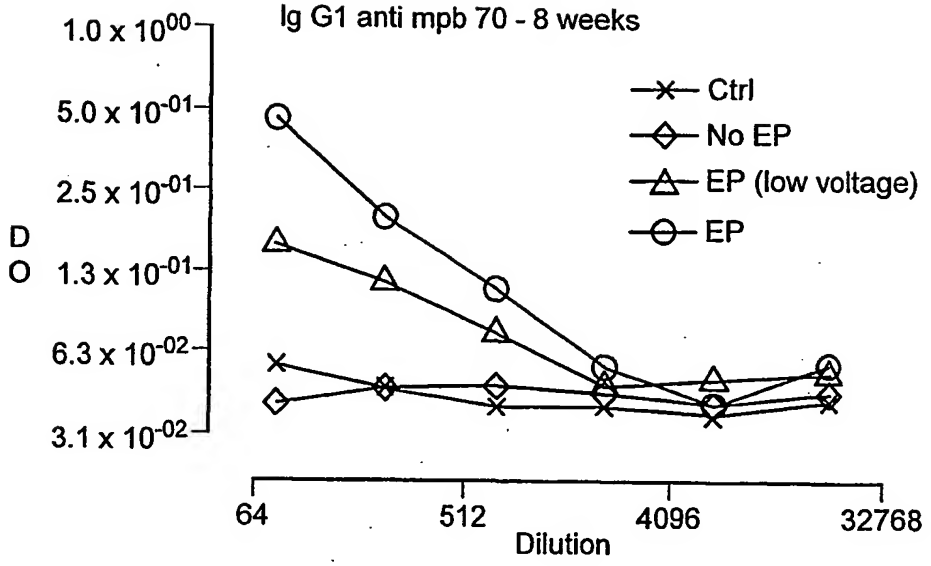

IgG2a anti MPB70 - 5 weeks
2.0 x 10^00
1.0 x 10^00
5.0 x 10^-01
2.5 x 10^-01
1.3 x 10^-01
6.3 x 10^-02
3.1 x 10^-02
D
O
85b +EP
MPB70 + mpb70 + EP
NaCl + mpb70 +EP
64
256
1024
4096
Dilution

FIG. 13B


Ig G1 anti mpb 70 - 8 weeks
1.0 x 10^00
5.0 x 10^-01
2.5 x 10^-01
1.3 x 10^-01
6.3 x 10^-02
3.1 x 10^-02
D
O
Ctrl
No EP
EP (low voltage)
EP
64
512
4096
32768
Dilution

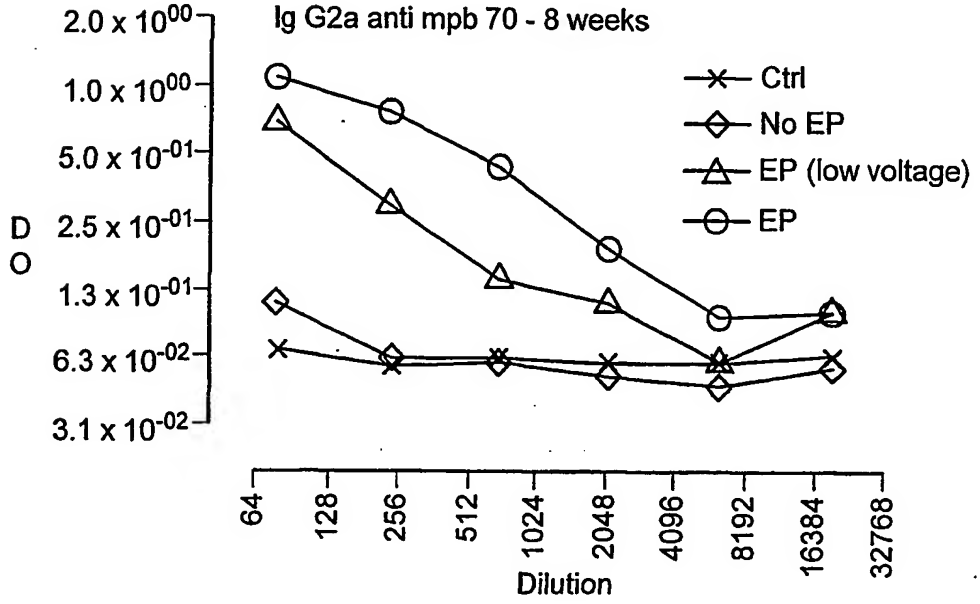

Ig G2a anti mpb 70 - 8 weeks
2.0 x 10^00
1.0 x 10^00
5.0 x 10^-01
2.5 x 10^-01
1.3 x 10^-01
6.3 x 10^-02
3.1 x 10^-02
D
O
Ctrl
No EP
EP (low voltage)
EP
64
128
256
512
1024
2048
4096
8192
16384
32768
Dilution

FIG. 14

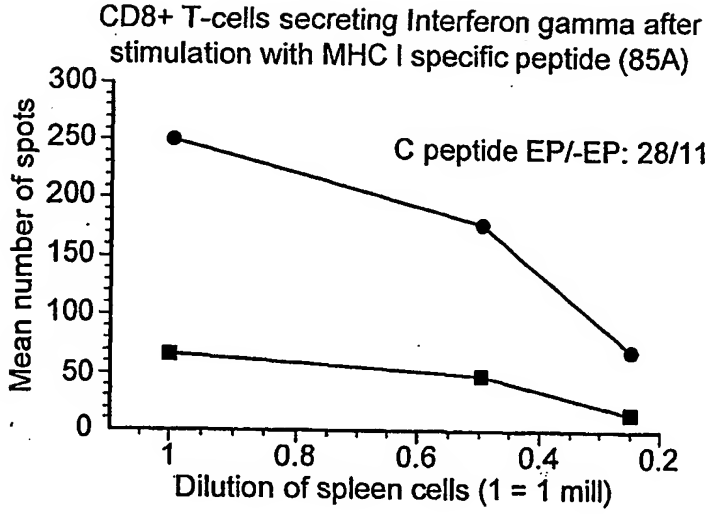


FIG. 15A

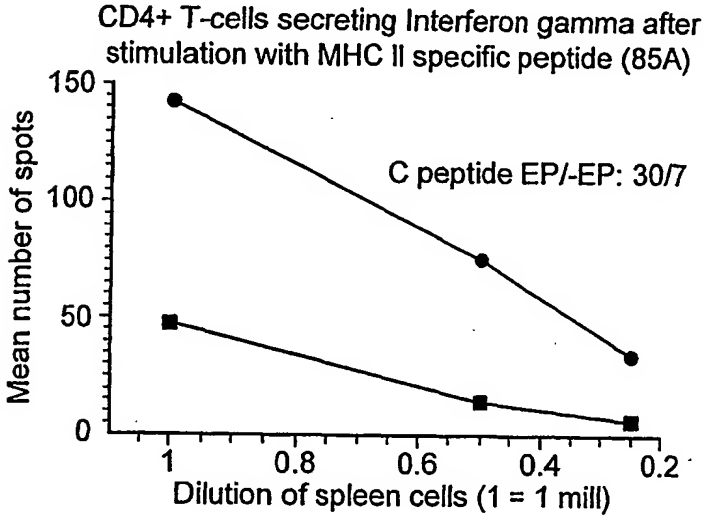


FIG. 15B

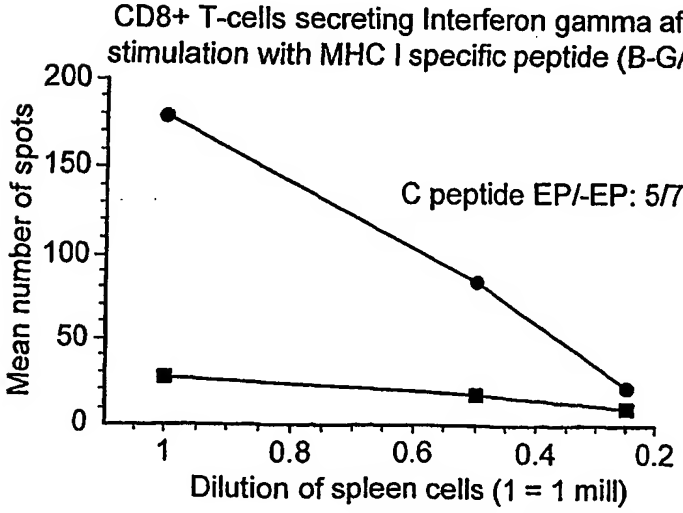
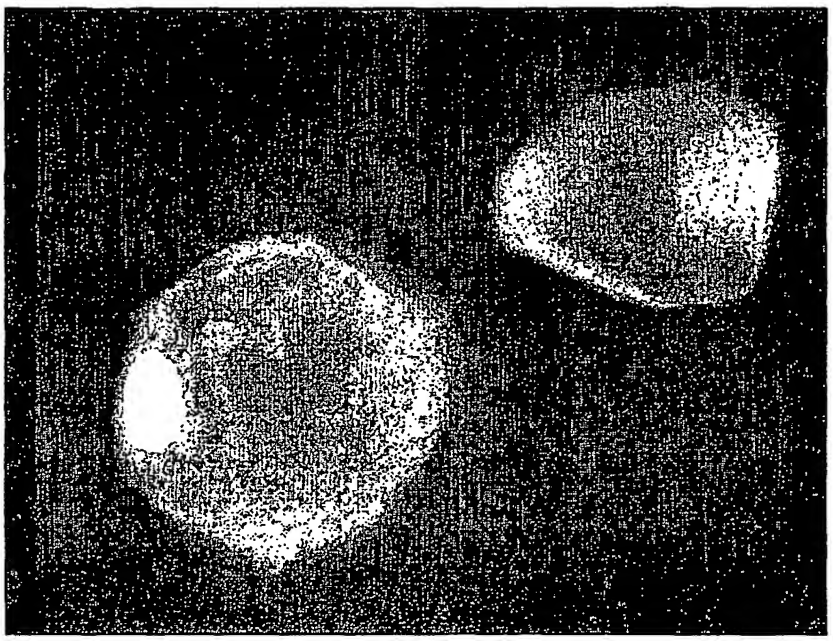
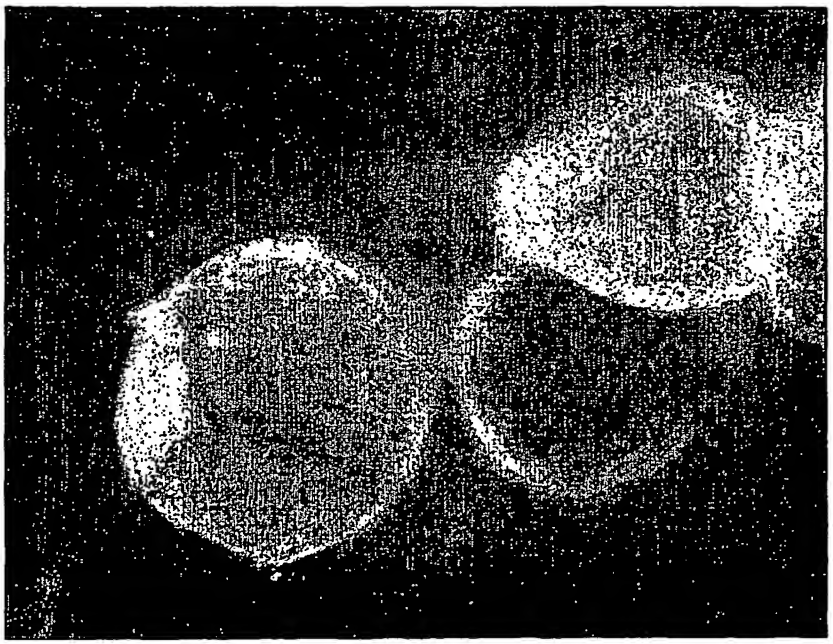


FIG. 15C

(A)



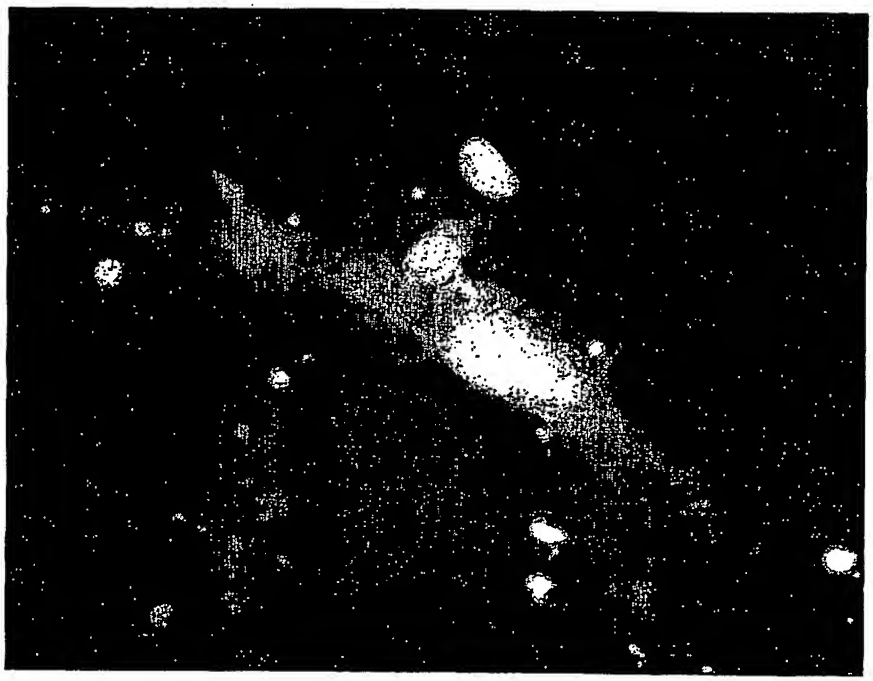
(B)



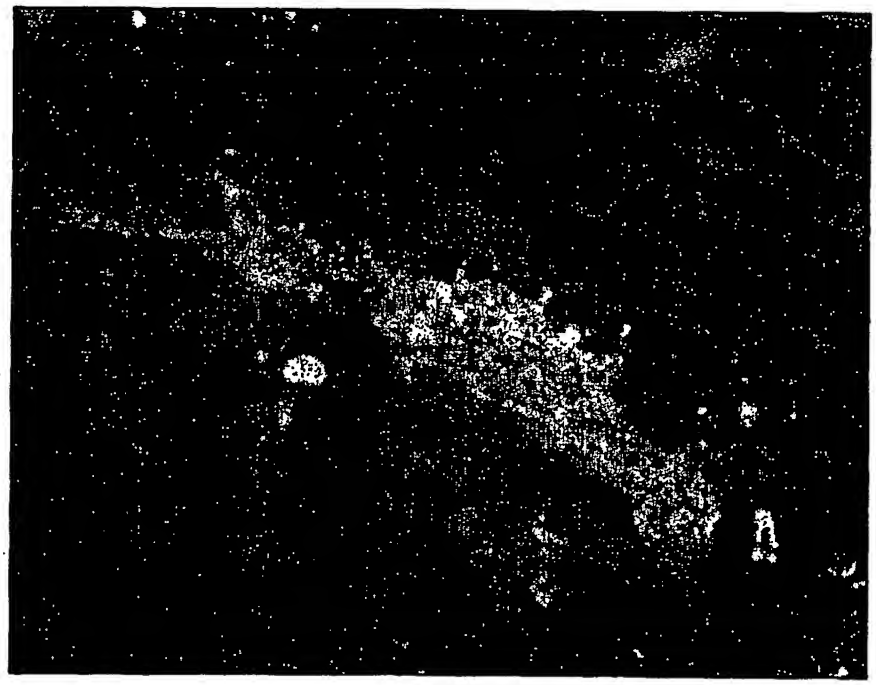
Adipocytes stained against S-100 (intracellular protein),
Adipocytes expressing GFP (A) and S-100 (B).

FIG. 16

(A)



(B)



Staining against thy-1 (membrane protein in the IgG superfamily, found in connective tissue cells)
Cell expressing GFP (A) and thy-1 (B).

FIG. 17

(A)

(B)

Staining against vimentin (intracellular protein found in connective tissue)
Cell expressing GFP (A) and vimentin (B).

FIG. 18

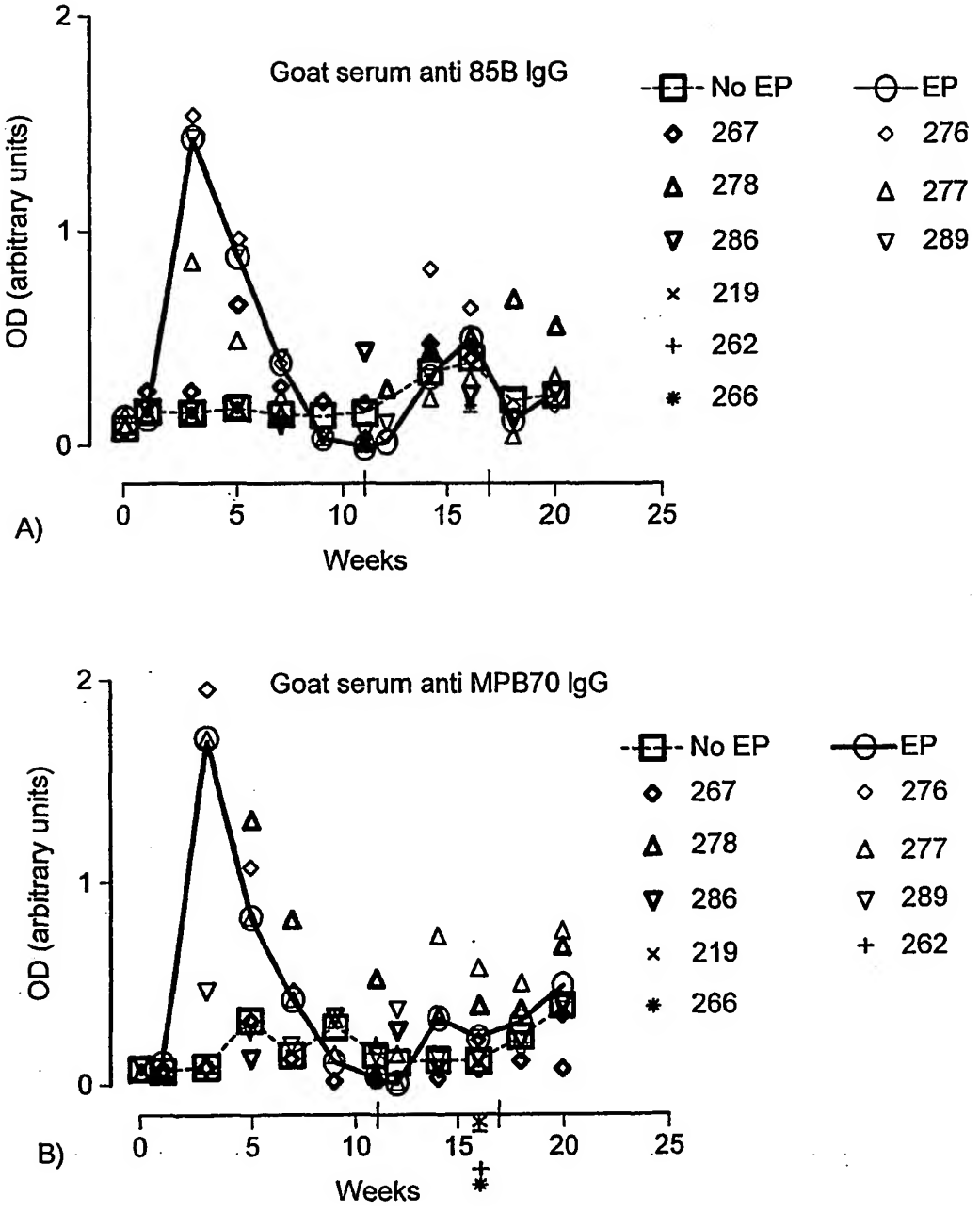


FIG. 19 Serum IgG levels in goats after DNA injection under local anesthetic, with and without electroporation (EP)

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(75) Inventors/Applicants *(for US only)*: MATHIESEN, Iacob [NO/NO]; Langlia 25, N-0854 Oslo (NO). TOLLEFSEN, Stig [NO/NO]; Institute of Physiology, IMBA, 1104 Blindern, N-0317 Oslo (NO).

(74) Agents: COCKBAIN, Julian et al.; Frank B. Dehn & Co., 179 Queen Victoria Street, London EC4V 4EL (GB).

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(54) Title: ELECTROPORATION FOR INTRODUCTION OF MOLECULES INTO CELLS

(57) Abstract: The invention provides a method of eliciting an immune system response in a mammal, which method comprises: injecting a first antigen into a first injection site in skeletal muscle in said mammal; optionally injecting a polynucleotide functionally encoding a second antigen into a second injection site in skeletal muscle in said mammal; positioning electrodes in said skeletal muscle such that current travelling between said electrodes passes through said first and/or second injection site; and electrically stimulating said skeletal muscle with an electrical current between said electrodes having a field strength in said skeletal muscle of from 10 to 300 V/cm whereby to assist in cellular uptake of said first antigen and/or said polynucleotide.

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A. CLASSIFICATION OF SUBJECT MATTER
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Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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EPO-Internal, BIOSIS, MEDLINE, WPI Data, PAJ, CHEM ABS Data

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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sitch, W

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Int ational Application No
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Information on patent family members

Int ·ational Application No
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